

**Induction of PAL during elicitation of
Capsicum frutescens Mill. cell cultures**

by

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
4CL	4-hydroxycinnamate:CoA ligase (EC 6.2.1.12)
A	adenine
abs.	absolute
ADP	adenosine diphosphate
AMPS	ammonium persulphate
ATP	adenosine triphosphate
AU	absorbance unit(s)
Bq	Becquerel(s)
GBq	Giga-Becquerel(s)
kBq	kilo-Becquerel(s)
MBq	Mega-Becquerel(s)
TBq	Tera-Becquerel(s)
BSA	bovine serum albumin
C	cytosine
ca.	approximately
CA3H	<i>para</i> -coumaric acid 3-hydroxylase
CA4H	<i>trans</i> -cinnamic acid 4-hydroxylase (EC 1.14.13.11)
CDTA	<i>trans</i> -1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
Ci	Curie
cm	centimetre(s)
cpm	counts per minute
cv.	cultivar
d	day(s)
DEAE	diethylaminoethyl
dist.	distilled
DNA	deoxyribonucleic acid
cDNA	complementary or copy DNA
hsDNA	herring sperm DNA
λ-DNA	DNA isolated from bacteriophage λ
dNTP	deoxynucleotide triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dpm	disintegrations per minute
DW	dry weight
<i>E.coli</i>	<i>Escherichia coli</i>
ed(s)	editor(s)
EDTA	ethylenediamine tetraacetic acid
EEO	electroendosmosis
<i>et al.</i>	<i>et alia</i>
FW	fresh weight
g	gram(s)
G	guanine
GAR	goat anti rabbit
h	hour(s)
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[ethanesulphonic acid])
HPLC	high performance liquid chromatography
IGSS	ImmunoGold™ Silver Staining
kb	kilobases (number of bases in thousands)

kg	kilogram(s)
kinetin	6-furfurylaminopurine
l	litre(s)
LSC	liquid scintillation counting
lysozyme	mucopeptide N-acetylmuramoyl hydrolase (EC 3.2.1.17)
M	molar
M+S	Murashige and Skoog
mA	milliampère(s)
MAU	milliabsorbance unit(s)
μCi	micro-Curie(s)
μg	microgram(s)
mg	milligram(s)
min	minute(s)
μl	microlitre(s)
ml	millilitre(s)
μm	micrometre(s)
μM	micromolar
mm	millimetre(s)
mM	millimolar
MOPS	3-(N-morpholino)propanesulphonic acid
nm	nanometre(s)
nM	nanomolar
OD	optical density
OLB	oligo labelling buffer
oligo(dT)	oligodeoxythimidylic acid
PAL	phenylalanine ammonia-lyase (EC 4.3.1.5)
PAS	4-aminosalicylic acid
PBS	phosphate buffered saline
pg	picogram(s)
pH	negative logarithm of the hydrogen ion concentration
poly(A)	polyadenylic acid
Ponceau S	3-hydroxy-4-(2-sulpho-4-[4-sulphophenylazo]-phenylazo-2,7-naphthalene disulphonic acid
ppm	parts per million, e.g. milligram per litre
PPO	2,5-diphenyloxazole
psi	pounds per square inch
PVC	polyvinyl chloride
PVP	polyvinyl pyrrolidone
R _f	relative electrophoretic mobility
RNA	ribonucleic acid
mRNA	messenger RNA
poly(A)+RNA	polyadenylated RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
RNase	ribonuclease
rpm	revolutions per minute
R _t	retention time
S+H	Schenk and Hildebrandt
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SE	standard error
sec	second(s)
SSC	saline sodium citrate
STE	saline Tris-EDTA

T	thymidine
TBE	Tris-borate EDTA
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TLC	thin layer chromatography
2D-TLC	two dimensional TLC
TMV	tobacco mosaic virus
TNS	tri- <i>isopropyl</i> naphthalene sulphonic acid
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
U	uracil
UV	ultraviolet (light)
V	volt(s)
v/v	volume per volume (as in %(v/v), which indicates the volume in ml per 100 ml total volume)
W	watt(s)
xg	times force of gravity

"... The enzyme responsible for this deceptively simple reaction is now named phenylalanine ammonia-lyase (EC 4.3.1.5) but often known affectionately as PAL ..."

Hanson and Havir (1979)

Abstract

This project was an investigation of the regulation of PAL induction, following elicitation of cell cultures of *Capsicum frutescens*, and its effects on phenylpropanoid metabolism.

It was shown that the response of PAL specific activity to elicitation increased throughout the culture cycle and consequently, stationary-phase cultures were used throughout the investigation. PAL activity in *Capsicum* cultures increased transiently after elicitation. Detailed investigation of the initial stages of elicitation showed that PAL activity was induced 40 min after the onset of the elicitor treatment and reached a maximum after *ca.* 8 h.

Capsicum PAL protein sub-units were identified and the molecular size was estimated to be 77 kD, the same size as PAL sub-units isolated from parsley leaves. *In vivo* labelling techniques showed that the transient induction of PAL specific activity following elicitation resulted from a combination of the general induction of protein synthesis and a specific induction of PAL protein synthesis. Additionally, the synthesis of a 32 kD protein, probably a chitinase precursor, was transiently induced with a lag phase of 4-8 h. A 70 kD polypeptide, possibly a degradation product of PAL sub-units, was recognised by anti-(parsley PAL) serum in samples from elicited and control cultures of *Capsicum frutescens* but not in *Petroselinum* samples.

PAL mRNA was identified and its molecular size was estimated to be 2.6 kb. Increased PAL mRNA steady state levels were detected following dilution and elicitation. The transient increase in PAL mRNA levels during elicitation followed the same pattern as PAL protein accumulation thus, indicating that the induction of PAL is regulated at the transcriptional level. Elicitation of *Capsicum* cultures increased PAL mRNA levels transiently resulting in *de novo* synthesis of PAL protein and thus, increased PAL activity.

Phenylpropanoid metabolism, as represented by the incorporation of [¹⁴C]phenylalanine into phenolic compounds, was studied during and after the transient induction of PAL activity. The majority of [¹⁴C]phenylalanine was incorporated into unknown phenolic compounds, including water soluble phenolic glycosides. Incorporation of [¹⁴C] was also detected in cinnamic acid, coumaric acid, and vanillin, putative free phenolic intermediates of capsaicin biosynthesis. The incorporation of radiolabel into vanillin during elicitor treatment followed the induction pattern of PAL activity but, [¹⁴C]vanillin was also detected after PAL activity had

returned to basal levels. The absolute amount of free phenolic compounds in the culture medium decreased following elicitation but "capsaicinoids" accumulated during the time course of elicitor treatment. Water soluble conjugated phenolic compounds, including 3,4-dimethoxycinnamoyl glycoside, increased transiently in *Capsicum* cells after elicitation, showing the same pattern of induction as PAL activity. It is proposed that these compounds are precursors of phenolics deposited in the cell wall as part of a defence response against pathogen attack. It is postulated that this is the major role of the changes in phenylpropanoid metabolism in cell cultures of *Capsicum frutescens* following elicitation.

1. INTRODUCTION

1.1 The synthetic potential of cultured plant cells

Plants exhibit a wide biosynthetic repertoire and are rich sources of valuable pharmaceuticals and other biologically active phytochemicals (DiCosmo and Misawa, 1985). Although, the trend has been towards chemical synthesis, plants still remain an important source of many of these compounds for a number of reasons (Yeoman *et al.*, 1980). The compounds may be difficult or costly to synthesise or may consist of complex mixtures, like essential oils, that cannot be constituted successfully. Isolation from natural sources can circumvent many of the regulations which must be satisfied before chemically synthetic compounds can be used commercially. Also, chemical synthesis may, depending on the compound, result in a mixture of isomers which cannot be separated on a commercial scale.

The majority of commercially important substances originate from plants grown in tropical and sub-tropical regions and the availability and cost of these materials is frequently affected by economic and political considerations in the countries of origin (Yeoman *et al.*, 1980). Field grown plants are also susceptible to attack by pathogens including fungi, bacteria, and viruses which can result in large crop losses. Conventional plant breeding techniques have achieved only limited success in overcoming problems of irregular ripening, variability in yield, and susceptibility to pathogens.

It is widely recognised that cultured plant cells represent a potential source of commercially valuable biochemicals (DiCosmo and Misawa, 1985). Furthermore, plant cell cultures provide a valuable tool with which to study aspects of metabolic control and cell differentiation in the absence of many of the complications of the intact organism (Yeoman, 1987). However, it is a characteristic feature of cultured plant cells that they usually fail to produce the levels of secondary metabolites found in the whole plant. Indeed, the spectrum of compounds produced is often quite different

from that in the plant, but it is the low yield of product which is the major barrier to commercial production of useful secondary metabolites by plant cells *in vitro* (Yeoman *et al.*, 1990).

1.2 Manipulations to improve product yield

1.2.1 Cell line selection

On the assumption that a large cell population may contain a few cells which, as a result of mutation or epigenetic change, constitutively produce a desired metabolite it is possible to carefully select cells that produce significantly larger amounts of secondary products than normal (Dix, 1990). However, such cell lines are likely to be genetically and metabolically unstable and continuous selection will be required to maintain productivity (Holden *et al.*, 1988c).

1.2.2 Culture conditions

It is widely accepted that most secondary metabolites are accumulated late in the culture cycle after cell division has ceased and this is associated with the process of cell differentiation (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1985a). Therefore, it seems vital to develop culture systems which can exploit this property of cultured plant cells in order to maximise the yield of a designated substance. Such culture manipulations include varying the concentration and balance of growth substances, increasing or decreasing the temperature, changing the level, quality or exposure to light, alternating the concentration of major mineral nutrients (usually nitrogen or phosphorus), supplying precursors to the product, altering the pH and varying the concentration of the carbon source (Yeoman, 1991). In general however, the manipulation of plant growth regulator levels in the media and other culture conditions have not allowed profitable commercialisation of plant cell culture metabolism. Notable exceptions are the production of shikonin by *Lithospermum erythrorhizon* cell cultures and

berberine by cultures of *Coptis japonica* (see Wink, 1987).

1.2.3 Cell immobilisation

The relationship between secondary metabolite production and the structural organisation or aggregation of cells in culture (Lindsey and Yeoman, 1983) led to the use of immobilised cells as a means of enhancing yields by artificially produced aggregates, in which metabolic intermediates common to primary and secondary pathways are preferentially used in the latter (Lindsey and Yeoman, 1984). The high degree of cell-cell contact, achieved by physical entrapment of plant cells in an inert matrix, may limit the rate of cell division (compared with suspended cells) and permit the establishment of gradients of nutrients and other factors which are important in the regulation of secondary metabolic activity (Lindsey and Yeoman, 1985b). Indeed, cells of *Capsicum frutescens*, immobilised in a matrix of polyurethane foam, produce significantly higher yields of capsaicin, the main pungent principle of chilli pepper fruits, than do freely suspended cells (Lindsey and Yeoman, 1984). Moreover, the immobilisation of intact plant cells provides a means by which the physical and chemical environment can be readily manipulated, and there is evidence to indicate that such culture technique could provide the basis for the industrial-scale production of valuable natural products, particularly secondary metabolites (Lindsey and Yeoman, 1984).

1.3 Elicitation as a means to study secondary metabolism

The effects of the above manipulations of culture conditions on the synthesis of products might be mediated by mechanisms such as enzyme repression and inhibition (DiCosmo and Misawa, 1985). If so, the removal of these regulatory blocks by manipulating pathway enzymes or genetic constitution may increase the enzymes of secondary metabolism several-fold. However, such manipulations have usually been per-

formed empirically with often unpredictable effects and, in fact, secondary metabolite production from cell culture to cell culture is so inconsistent that it is obvious that the regulation of secondary metabolism is poorly understood (DiCosmo and Misawa, 1985).

It is widely accepted, that the microbial invasion of intact, whole plants and treatment of cell cultures with microbial elicitors leads to the synthesis of antimicrobial secondary metabolites. The mechanisms by which elicitors affect secondary metabolism remains to be clearly defined and much current research activity is directed towards the understanding of elicitor-mediated biochemical processes (Barz *et al.*, 1991; Bernards and Ellis, 1991; Bernards *et al.*, 1991; Bhandal and Paxton, 1991; Douglas *et al.*, 1991; Edwards *et al.*, 1991; Felix *et al.*, 1991a,b; Gleitz *et al.*, 1991; Godiard *et al.*, 1991; Gowri *et al.*, 1991; Graham and Graham, 1991; Lozoya *et al.*, 1991; Marinelli *et al.*, 1991; Matern, 1991; Messner *et al.*, 1991; Milat *et al.*, 1991; Nef *et al.*, 1991; Niemann *et al.*, 1991; Oku, 1991; Paiva *et al.*, 1991; Saimmaime *et al.*, 1991; Schmitt *et al.*, 1991; Sumaryono *et al.*, 1991; Ward *et al.*, 1991). Indeed, elicitation has proved to be a powerful research tool to help explain the regulation of secondary metabolites in plants (see Hahlbrock and Scheel, 1989) and several of the molecular probes used to localise pathway activities in whole plant tissues could not have been generated easily from starting material other than elicited cell cultures (e.g. Cramer *et al.*, 1989). Elicitation can influence the flux of metabolites in biosynthetic pathways of secondary products, often via the induction of specific enzymes thus, facilitating the molecular analysis of the particular pathway (Hahlbrock and Scheel, 1989).

1.3.1 Elicitors

Compounds from pathogens have been found to cause the same response in plants as the pathogen itself (Wolters and Eilert, 1983). These compounds are termed elici-

tors (Keen *et al.*, 1972). The so-called endogenous or constitutive elicitors from the host plant (Dixon *et al.*, 1989) together with elicitors derived from microorganisms comprise the group of biotic elicitors.

Elicitor molecules are often oligosaccharides and indeed, Cheong *et al.* (1991) have shown that chemically synthesised oligo- β -glucosides (hexamer to decamer) possessed very high elicitor activity in soybean coyledons. They also reported that a branched trisaccharide at the non-reducing end of the oligosaccharides is essential for maximum elicitor activity, measured as induced phytoalexin accumulation.

Physical and chemical stresses like UV irradiation, exposure to cold or heat, ethylene, fungicides, antibiotics, salts of heavy metals, or high salt concentrations, which can also induce product accumulation, are sometimes referred to as abiotic elicitors (Eilert, 1987). However, different modes of action for biotic and abiotic elicitors have been postulated by Yoshikawa (1978). In this thesis the term elicitor denotes a compound only of biological origin, while other treatments, including UV light, heavy metals and dilution of cultures, are referred to as abiotic stress.

1.3.2 Signals and responses in plant cell-elicitor interactions

Although, the exact mode of action of elicitors is not yet fully understood, a common working hypothesis (Lamb *et al.*, 1989) is that mechanisms involved in activation of defence genes following molecular recognition in the early stages of an incompatible interaction (host resistant, pathogen avirulent) are closely related to those operating in cell suspension cultures treated with fungal elicitor. In contrast, stress activation by abiotic elicitors such as HgCl_2 is likely to be an appropriate model for activation of defence genes in response to cell trauma and death at the onset of lesion formation in the later stages of a compatible interaction (host susceptible, pathogen virulent).

Evidence from current research on elicitation (see above) suggests common, or at

least very similar, mechanisms leading to the induction of secondary metabolism and a basic concept is summarised in Fig. 1.3.1 which shows a simplified model of plant cell-pathogen interactions. The model is based upon a gene-for-gene relationship between each resistance/susceptibility gene locus which has been suggested in 43 different plant-pathogen interactions (see Gabriel and Rolfe, 1990) including viruses, bacteria, fungi, insects, and nematodes, and follows, in part, a similar model proposed by Lamb *et al.* (1989).

An initial surface recognition (Staples *et al.*, 1986) by the pathogen follows a possible activation of a range of basic pathogenicity factors (Lamb *et al.*, 1989) that may include attachment (Hamer *et al.*, 1988), infection-associated differentiation (Staples *et al.*, 1986), nutrient assimilation, toxin production (Panopoulos and Peet, 1985) and the release of cuticle and wall degrading enzymes (Kolattukudy, 1985). Also, wound metabolites are thought to activate pathogenicity genes which regulate the expression of avirulence (*AVR*) genes leading to the production of race specific elicitors (Keen, 1986). The genetic properties of the *AVR* genes characterised to date are in accord with the predictions for the gene-for-gene hypothesis (Lamb *et al.*, 1989). Thus, cloned bacterial *AVR* genes confer on the recipient bacterium the host-cultivar-specific avirulence phenotype of the race from which the gene was isolated, but do not impart the host-cultivar-specific virulence phenotypes of the donor, confirming the dominance of the avirulence genotype (Staskawicz *et al.*, 1984). Moreover, each of five *AVR* genes from *Xanthomonas campestris* pv. *malvacearum* specifically matches, on a gene-for-gene basis, individual resistance genes in cotton (Gabriel *et al.*, 1986).

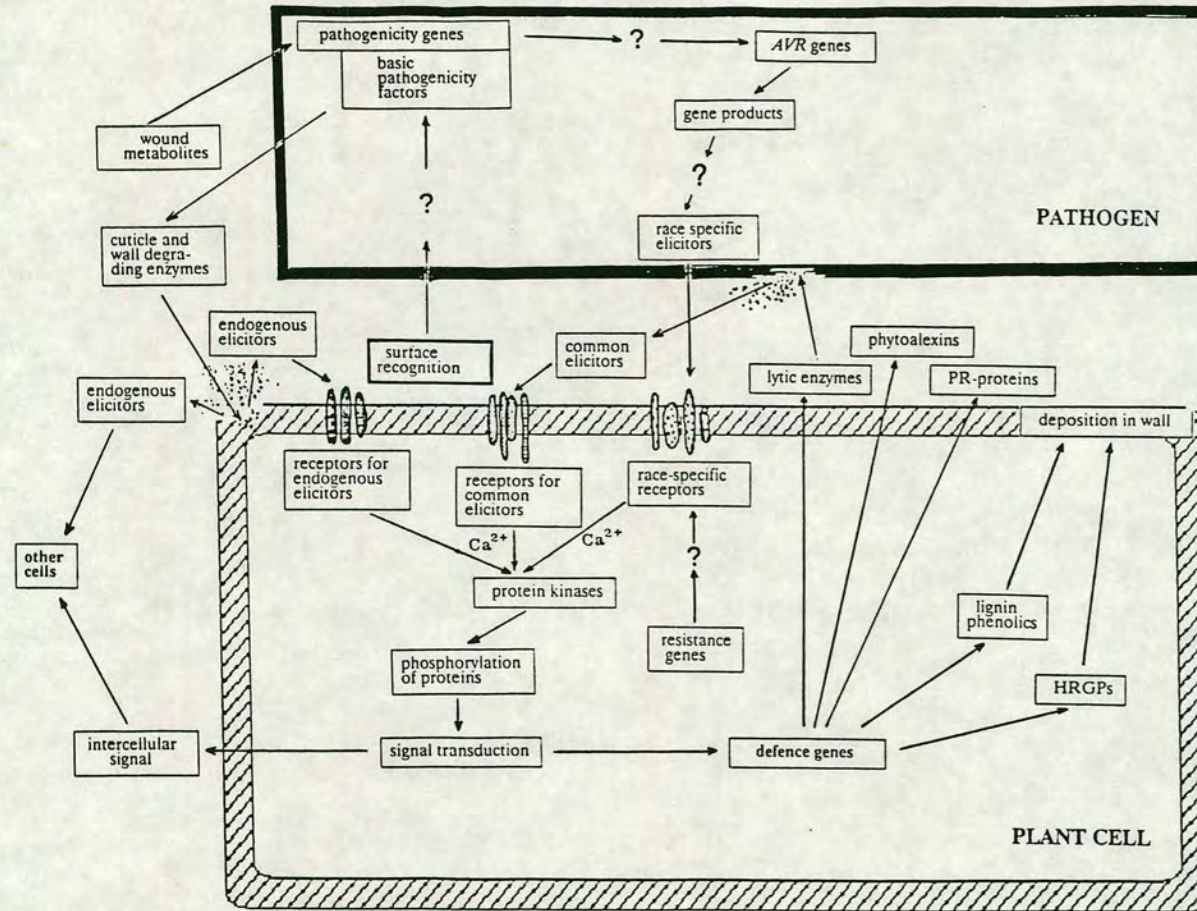


Figure 1.3.1 Signals and transduction in plant cell-pathogen interactions. The text deals with items approximately from left to right.

Three classes of receptors have been postulated for the recognition of elicitor molecules (Gabriel and Rolfe, 1990). Firstly, a highly conserved class of receptor molecules that respond to endogenous elicitors which may be released from the plant cell wall (Davies and Hahlbrock, 1987) by wounding and cell wall degrading enzymes (Tenhaken and Barz, 1991). Endogenous elicitors may sensitise the plant cell to low levels of exogenous elicitor molecules (Lamb *et al.*, 1989) and transmit the signal to other cells thus, contributing to a systemic response against pathogen attack. The second class of receptor molecules responds to most common exogenous elicitors found conserved in fungi, such as chitin and glucanes (Cheong *et al.*, 1991; Kurosaki *et al.*, 1988), which might be released due to the activity of lytic enzymes like chitinases and glucanases (Ward *et al.*, 1991; Hughes and Dickerson, 1991; Hedrick *et al.*, 1988) that are synthesised by the plant cell as part of the defence response. The third class is a group of specific receptors that are poorly conserved and continually generated *de novo* (Gabriel and Rolfe, 1990). These transmembrane proteins are encoded by specific resistance genes and, according to the ion-channel defence model (Gabriel *et al.*, 1988), are capable of being opened into ion-channels when bound by specific or semi-specific elicitors. Therefore, the initial signal transduction seems to be due to electrolyte fluxes, especially calcium (Knight *et al.*, 1991; Stab and Ebel, 1987), which, in case of a hypersensitive response, can lead to depolarisation of membranes and cell death (Gabriel and Rolfe, 1990).

The sudden increase in cytoplasmic calcium activates protein kinases leading to the phosphorylation of a number of proteins (Felix *et al.*, 1991b). The signal transduction chain may further involve *cis*-acting elements (Harrison *et al.*, 1991) or alternatively, the modulation of *trans*-acting nuclear regulatory proteins (Lamb *et al.*, 1989). Also, the operation of more than one intracellular transduction pathway for defence gene activation (Hamdan and Dixon, 1987) has to be considered.

Plants elaborate a number of inducible defence responses following elicitor treat-

ment, mechanical damage, or microbial attack (see Bolwell *et al.*, 1991; Lamb *et al.*, 1989). These responses include synthesis of antimicrobial phytoalexins; reinforcement of cell walls by deposition of callose, lignin, and related wall-bound phenolics, and accumulation of hydroxyproline-rich glycoproteins (HRGPs); and production of proteinase inhibitors and lytic enzymes such as chitinases and glucanases, which can attack microbial cell walls (Dixon, 1986). In addition, an empirical class of so-called pathogenesis-related (PR) proteins accumulate in response to elicitation. However, some of these PR proteins have been identified as proteinase inhibitors or extracellular forms of chitinase and glucanase (Kombrink *et al.*, 1988; Kauffmann *et al.*, 1987; Legrand *et al.*, 1987). Direct evidence for the role of inducible defence responses in expression of disease resistance comes from the ability of L- α -aminooxy-3-phenylpropionic acid (AOPP), a potent and specific inhibitor of phenylalanine ammonia-lyase, the first enzyme in the phenylpropanoid biosynthetic pathway, to make soybean seedlings susceptible to normally avirulent races of *Phytophthora megasperma* (Moesta and Grisebach, 1982).

1.4 Capsaicin biosynthesis

Empirically-derived procedures to enhance metabolite yield in plant cell cultures (nutrient limitation, cell immobilisation, precursor feeding, etc.), while successful, have given little indication as to how the metabolism of the cells is affected by these manipulations. A more detailed knowledge of biosynthetic pathways and of how the modification of culture conditions affects product yield will not only provide a better understanding of cell culture metabolism but will also enable a more direct approach to be adopted in the manipulation of plant cell and tissue cultures to produce higher yields (Hall *et al.*, 1987).

Elicitation of plant cell cultures, which has proved to be a valuable tool for the study of secondary metabolism (see Hahlbrock and Scheel, 1989), was used in this

study to investigate the regulation of secondary metabolite production because, elicitation often triggers a rapid "switch-on" of enzymes involved and thus, facilitates the molecular analysis of biosynthetic pathways.

The accumulation of capsaicin, the hot flavour compound of chilli pepper fruit, in cell cultures of *Capsicum frutescens* has been used as an experimental system to study the regulation of secondary metabolite production. Capsaicin biosynthesis in cell cultures is of particular interest in studying the relationship between primary and secondary metabolism as the accumulation of this secondary product is under tight developmental control in the whole plant (Holden *et al.*, 1987). Indeed, capsaicin is only accumulated in the epidermal cells of the placental tissue of chilli pepper fruit during a relatively short period in the later stages of fruit development and the pathway is not expressed in roots, shoots, or leaves (Holden *et al.*, 1987). However, suspension cultures derived even from stem tissue will synthesise capsaicin when given the appropriate culture conditions (Lindsey and Yeoman, 1984), indicating that the pathway is present and potentially operative in all *Capsicum* cells.

Five different "capsaicinoids" have been reported in chilli pepper fruits (Iwai *et al.*, 1979). These are capsaicin, homocapsaicin, dihydrocapsaicin, homodihydrocapsaicin, and nordihydrocapsaicin (Fig. 1.4.1). Structurally, they differ only in the presence or absence of the double bond at the 6 position on the acyl side chain or the length of the acyl moiety (9 to 11 carbon atoms). Capsaicin and dihydrocapsaicin are normally the major components (Bennett and Kirby, 1968) and represent at least 95% of the total "capsaicinoid" content.

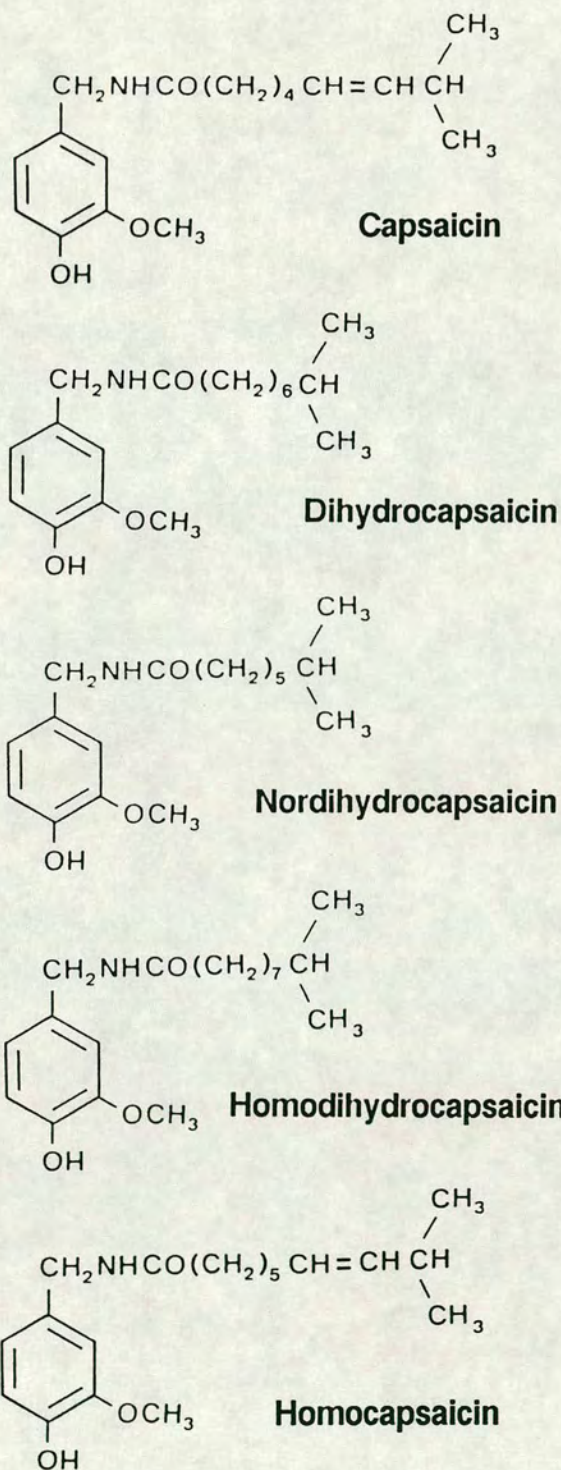


Figure 1.4.1 Structures of five "capsaicinoids" which occur naturally.

The biochemical pathway which leads to capsaicin has not been fully elucidated but the most likely steps are presented diagrammatically in Fig. 1.4.2. The pathway has two distinct branches (Bennett and Kirby, 1968; Leete and Loudon, 1968): the aromatic moiety is derived from phenylalanine while the acyl component is derived from valine. Studies on the intermediates of capsaicin biosynthesis have concentrated on the aromatic branch of the pathway because it contains the core reactions of general phenylpropanoid metabolism (Hanson and Havir, 1979). Additionally, Holden *et al.* (1987) reported that yields of capsaicin from suspension cultures can be increased by feeding with phenylalanine but not by feeding with valine.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the first step in the phenolic branch of capsaicin biosynthesis, the formation of *trans*-cinnamic acid from L-phenylalanine. Therefore, PAL forms a junction between primary and secondary metabolism and consequently, is frequently referred to as a "bridging enzyme" or the first committed enzyme of phenylpropanoid metabolism (Hanson and Havir, 1979;1981). PAL was first described by Koukol and Conn (1961) and since then it is probably the most studied enzyme concerned with secondary metabolism in plants (see Camm and Towers, 1977). Indeed, PAL is considered to be a key enzyme in several systems and increases in product yield are often correlated with changes in PAL activity (Jones, 1984). The regulatory role of PAL in capsaicin synthesis seems more complex, Holland (1989) has shown that PAL does not have a key regulatory function in capsaicin synthesis in *Capsicum* fruit, whereas studies on elicited cell cultures by Holden *et al.* (1988a,b) indicated that capsaicin accumulation is correlated with increased PAL activity. Hence, elicited *Capsicum* cultures have been used in this study to further investigate the regulatory role of PAL in phenylpropanoid metabolism.

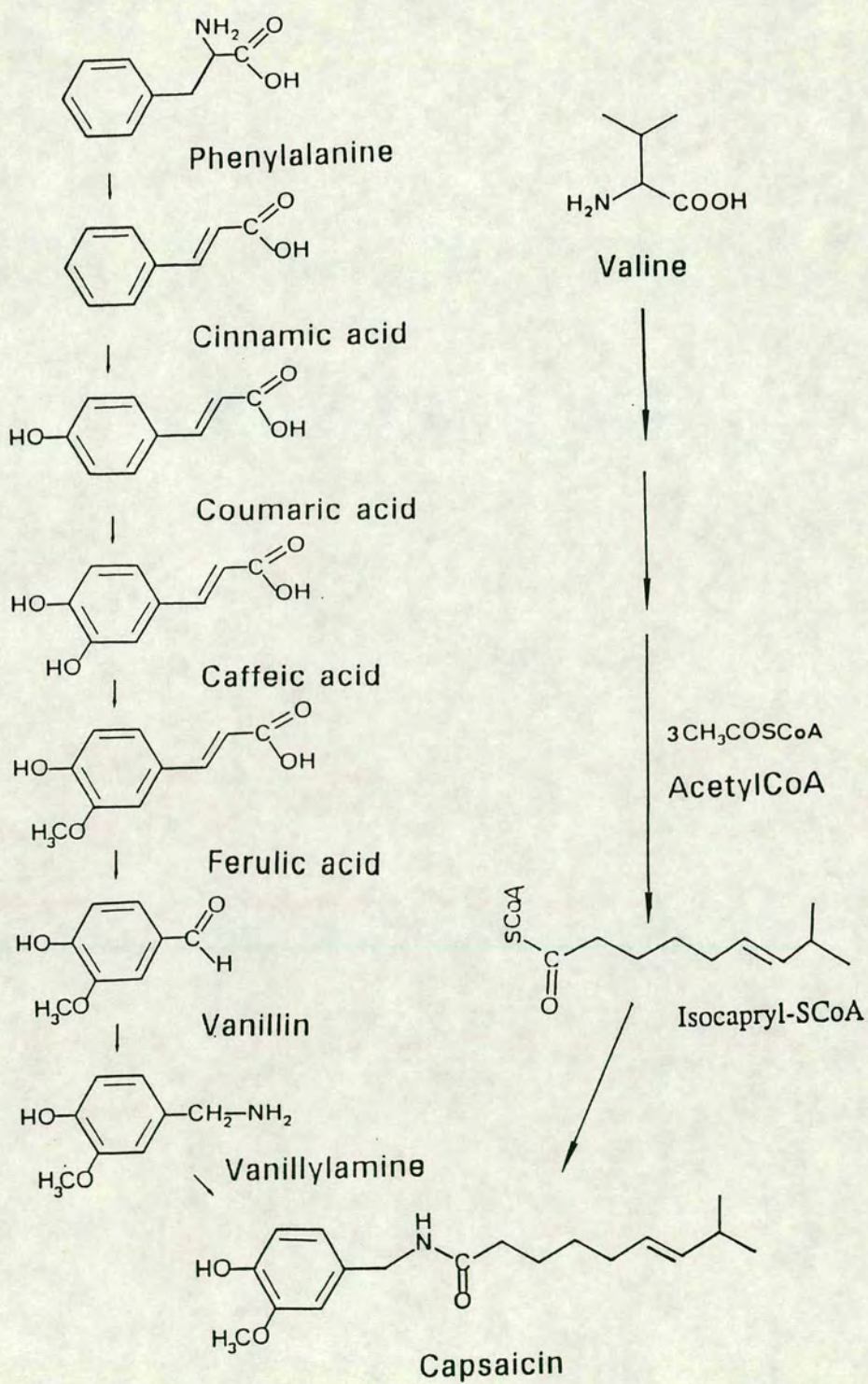


Figure 1.4.2 Proposed biosynthetic pathway of capsaicin.

1.5 Aims and objectives

The aim of this investigation was the analysis of the regulation of PAL induction following elicitation of cell cultures of *Capsicum frutescens* and the study of its effects on phenylpropanoid metabolism. The following objectives were identified.

- 1) The detailed description of the effects of elicitor treatment on PAL activity in cell cultures of *Capsicum frutescens*, especially the timing and transiency of the induction after the addition of the fungal elicitor.
- 2) The identification and characterisation of *Capsicum* PAL protein and the subsequent correlation of possible *de novo* synthesis of PAL protein sub-units with increases in PAL activity following elicitation.
- 3) The identification and characterisation of *Capsicum* PAL mRNA and the elucidation of PAL induction at a transcriptional level, by following PAL mRNA steady state levels during elicitor treatment of *Capsicum* cell cultures.
- 4) The qualitative and quantitative analysis of the effects of PAL induction following elicitation on the accumulation of free and conjugated soluble phenolic compounds in cell cultures of *Capsicum frutescens*.
- 5) The correlation of the pattern of PAL induction following elicitor treatment of *Capsicum* cultures with the incorporation of radioactively labelled phenylalanine into free phenolic intermediates of capsaicin biosynthesis and into water soluble conjugated phenolic compounds.

2. MATERIALS AND METHODS

2.1 Materials

The plant species used in this investigation was *Capsicum frutescens* Mill. cv. Cayenne (Fig. 2.1.1.). Cell cultures were initiated from mature, greenhouse grown plants which were previously raised from seeds obtained from Wm. K. McNair (seed merchant, Edinburgh, U.K.). Minimum temperature in the greenhouse was 16°C with a 16 h day. The light intensity was variable due to environmental factors and was supplemented with light from 400 W mercury vapour lamps.

Gliocladium deliquescens Sopp was obtained from CAB International Mycological Institute, Culture Collection, Kew (Cat.No. 101525).

Parsley plants (*Petroselinum hortense* Hoffm.) were a kind gift from the College of Agriculture, Edinburgh University.



Figure 2.1.1 Greenhouse grown plant of *Capsicum frutescens* Mill. cv. Cayenne

2.2 Sterilisation techniques for cell and tissue cultures

2.2.1 Surface sterilisation of explants

The external surface of plants supports a rich microflora which must be removed by surface sterilisation before aseptic excision of tissue explants (Thomas and Davey, 1975).

The cut ends of stem segments of *Capsicum frutescens* were sealed with paraffin wax and placed in a Pyrex glass tube (25 mm

diameter x 150 mm length) with both ends sealed with a double layer of muslin (Reinert and Yeoman, 1982). The explants were submerged in 70% (v/v) ethanol for 1 min to kill bacteria and to remove waxes from the epidermal cell layer in order to enable the surface of the material to be wetted by the sterilant. The explants were then immersed in sodium hypochlorite solution containing 2-2.8% available chlorine for 20 min. The sterilant was removed by washing the plant material in several changes of sterile dist. water.

All cell culture manipulations were carried out in a laminar air-flow cabinet, the work surface of which was swabbed with abs. ethanol before and after use. Air, forced under pressure through a bacterial filter, flows over the working bench at a uniform rate. Instruments, while not in use, were stored in ethanol, and flamed immediately prior to use.

2.2.2 Sterilisation of culture media

Liquid medium was dispensed into 250 ml conical (Erlenmeyer) flasks and these were covered with a double layer of aluminium foil. The flasks, each containing 50 ml medium, were sterilised by autoclaving for 20 min at 121°C (15 psi steam pressure).

Medium supplemented with agar was autoclaved in larger flasks, cooled to 50°C (water bath), and dispensed into Petri dishes under aseptic conditions. Where glass jars (250 ml) were used for solid cultures, the medium (50 ml) supplemented with agar was autoclaved in the jars which were covered with a double layer of aluminium foil during the sterilisation process. The medium solidified in the glass jars upon cooling.

Medium supplemented with ampicillin was autoclaved without the antibiotic and allowed to cool to 50°C (water bath) before filter (0.2 µm pore size) sterilised ampicillin was added under aseptic conditions.

2.3 Cell culture techniques

2.3.1 *Capsicum frutescens*

Callus cultures (Fig. 2.3.1) were initiated from stem segments of mature, greenhouse grown plants of *Capsicum frutescens* Mill. cv. Cayenne. The segments were surface sterilised as described in section 2.2.1 and sliced under aseptic conditions. Four slices (*ca.*

10 mm in length) per 9 cm Petri dish were plated onto M+S medium supplemented with 0.5 ppm 2,4-D, 0.1 ppm kinetin and 3% sucrose. Constituents of the culture media used are listed in Table 2.3.1. Solid medium for callus contained 1% agar (Agar No.1, Oxoid, U.K.). Developing callus was sub-cultured onto fresh medium every two weeks.



Figure 2.3.1 Callus culture of *Capsicum frutescens* grown on S+H medium supplemented with 2 ppm CPA, 0.5 ppm 2,4-D, 0.1 ppm kinetin, 3% sucrose, and 1% agar.

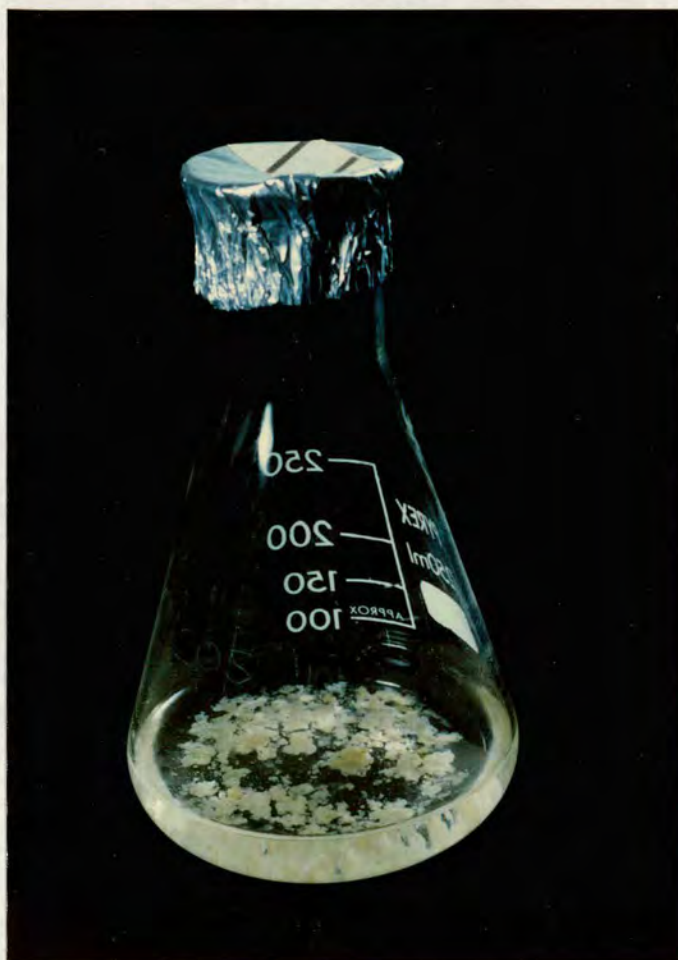


Figure 2.3.2 Suspension culture of *Capsicum frutescens* maintained on S+H medium supplemented with 2 ppm CPA, 0.5 ppm 2,4-D, 0.1 ppm kinetin and 3% sucrose.

After two sub-cultures *Capsicum frutescens* callus was transferred onto S+H medium (Table 2.3.1) supplemented with 2 ppm CPA, 0.5 ppm 2,4-D, 0.1 ppm kinetin and 3% sucrose. More friable calli developed on S+H, compared to M+S where growth was slow and the calli formed hard aggregates. Subsequently, fast growing friable cultures were selected for the initiation of suspension cultures.

Table 2.3.1 Constituents of Schenk and Hildebrandt (S+H) medium (Schenk and Hildebrandt, 1972) and Murashige and Skoog (M+S) medium (Murashige and Skoog, 1962). Both media were supplied in powder form by Imperial Laboratories (M+S Product No. 9-300-50, S+H Product No. 9-650-35).

Constituents	Concentration in media [mg·l ⁻¹]	
	M+S	S+H
CaCl ₂ ·2 H ₂ O	440.0	200.0
CoCl ₂ ·6 H ₂ O	0.025	0.1
CuSO ₄ ·5 H ₂ O	0.025	0.2
FeNaEDTA	36.7	-
FeSO ₄ ·7 H ₂ O	-	15
H ₃ BO ₃	6.2	5.0
KH ₂ PO ₄	170.0	-
KI	0.83	1.0
KNO ₃	1900.0	2500.0
MgSO ₄ ·7 H ₂ O	370.0	400.0
MnSO ₄ ·4 H ₂ O	22.3	10.0
Na ₂ EDTA	-	20.0
Na ₂ MoO ₄ ·2 H ₂ O	0.25	0.1
NH ₄ H ₂ PO ₄	-	300.0
NH ₄ NO ₃	1650.0	-
ZnSO ₄ ·7 H ₂ O	8.6	1.0
Glycine	2.0	-
myo-Inositol	100.0	1000.0
Nicotinic acid	0.5	5.0
Pyroxidine-HCl	0.5	0.5
Thiamine-HCl	0.1	5.0

Suspensions cultures (Fig. 2.3.2) were started by inoculation of 50 ml S+H medium (without agar) with *ca.* 2 g FW friable *Capsicum* callus. Suspensions were routinely sub-cultured every 28 d, unless stated otherwise in the text. A sterile sieve spoon was used to

transfer 1-2 g FW of cells into 50 ml of fresh liquid medium. The culture conditions for callus and suspension cultures are described in Table 2.3.2. Fine suspensions with small cell aggregates were selected, larger aggregates were excluded by filtering through nylon mesh (pore size 600 μm) under sterile conditions for six subsequent sub-cultures. Callus cultures were kept as backup on agar in 250 ml glass jars and sub-cultured every 8 weeks.

Table 2.3.2 Culture conditions for *Capsicum frutescens* callus and suspension cultures.

Temperature	$24 \pm 1^\circ\text{C}$
Photon flux	$25 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$
Light source	Compton warmwhite fluorescent
Culture dishes (callus culture)	9 cm polystyrene Petri dishes (Cell Cult) containing <i>ca.</i> 20 ml medium, sealed with Parafilm™ to prevent desiccation
	250 ml glass jars containing <i>ca.</i> 50 ml medium, bottom half of 5 cm Petri dish as a lid, sealed with Parafilm™ to prevent desiccation
Culture flasks (suspension culture)	250 ml conical (Erlenmeyer) flasks containing 50 ml medium, double layer aluminium foil as a seal
Liquid culture agitation	Continuous rotation in a horizontal plane, 98 rpm, 1.3 cm amplitude

2.3.2 *Gliocladium deliquescens*

Freeze dried cultures of *Gliocladium deliquescens* Sopp were obtained from CAB International Mycological Institute, Culture collection, Kew (Cat.No. 101525). The lyophilised cultures were re-hydrated and streaked onto one of three nutrient media. Bacto™ Oatmeal agar (Difco Laboratories, USA), Bacto™ Corn meal agar (Difco Laboratories, USA) or S+H medium (Table 2.3.1) supplemented with 2 ppm CPA, 0.5 ppm 2,4-D, 0.1 ppm kinetin, 3% sucrose, and 1% agar. Growth was fastest on S+H medium and this was subsequently used for further sub-cultures.

One week after inoculation of the agar plates the cultures turned dark green (Fig. 2.3.3) which indicated the *en masse* appearance of conidia (Fig. 2.3.4). Culture conditions are outlined in Table 2.3.3.

Table 2.3.3 Culture conditions for *Gliocladium deliquescens* Sopp solid cultures

Temperature	25 ± 1°C
Photon flux	36 µmol·m ⁻² ·sec ⁻¹
Light source	Thorn Universal white (6 x 30 W)
Culture dishes	9 cm polystyrene Petri-dishes (Sterilin) containing <i>ca.</i> 15 ml medium, sealed with Parafilm™ to prevent desiccation

2.3.3 *Escherichia coli*

An *E. coli* strain MC 1061 stab culture containing a 1700 bp long parsley PAL cDNA (pcPAL 35B) was a kind gift from Prof. Dr. K. Hahlbrock (Max-Planck-Institute, Cologne, Germany). The cDNA was cloned into the *EcoRI* site of the vector pBS M13+ which carries the gene for ampicillin

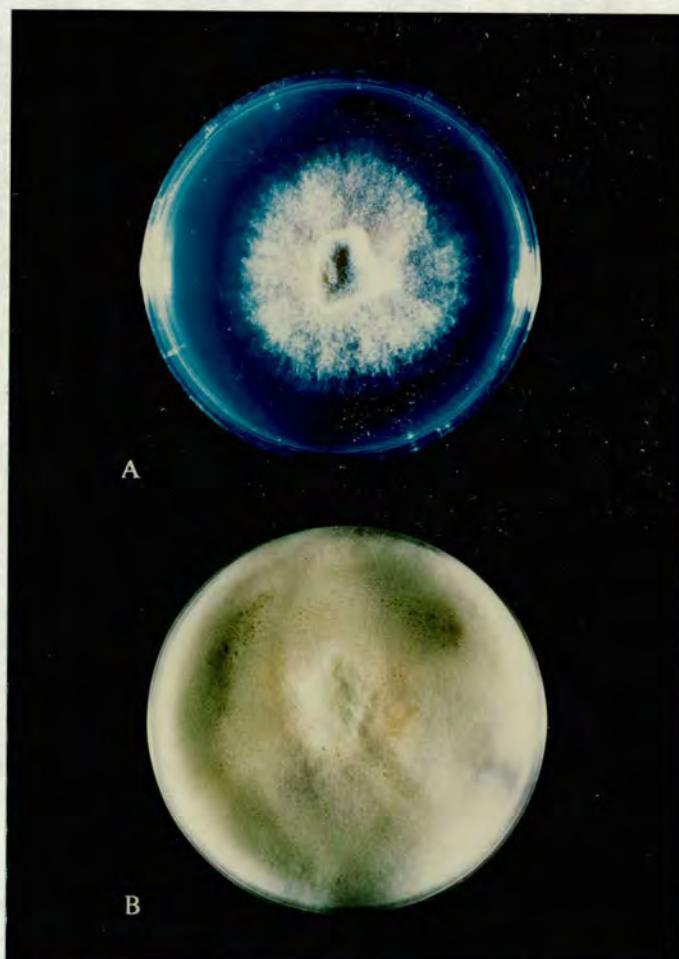


Figure 2.3.3 Solid cultures of *Gliocladium deliquescens* Sopp. A) 48 h after inoculation. Mycelium starts spreading from the inoculant (centre) B) 1 week after inoculation. Mycelium covers the entire Petri dish. The conidia *en masse* are green in colour.

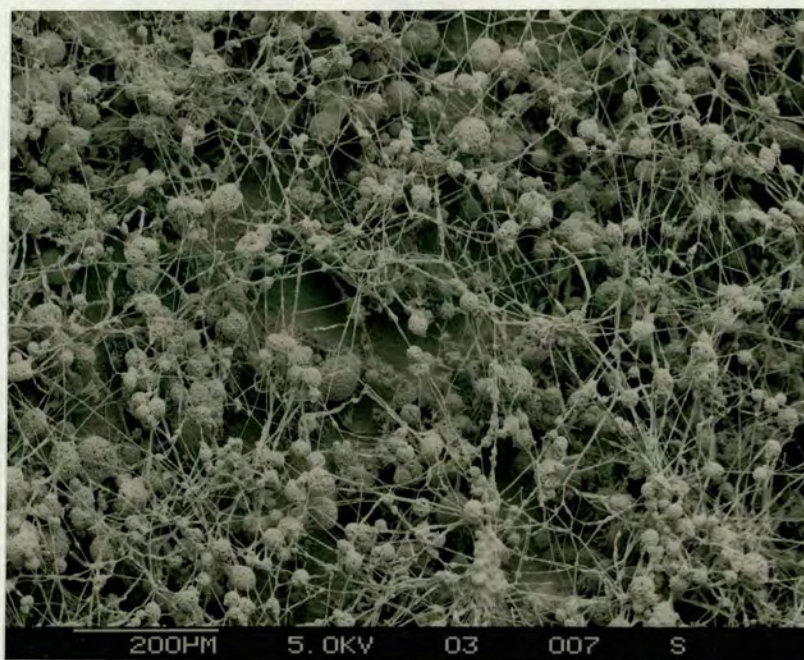


Figure 2.3.4 Scanning EM photograph of *Gliocladium deliquescens* culture after the mycelium covered the entire surface of the Petri dish

resistance. Ampicillin is a derivative of penicillin that kills growing cells by interfering in bacterial cell wall synthesis. The resistance gene specifies a periplasmic enzyme, β -lactamase, which cleaves the β -lactam ring of the antibiotic (Maniatis *et al.*, 1982).

Single colonies were obtained by streaking bacteria adhering to a platinum transfer loop onto a segment of a Petri dish (Fig. 2.3.5) containing 20 ml of LB-medium (Table 2.3.4). Because each colony is the progeny of a single bacterial cell, genetically homogeneous cultures were obtained by touching the colony with a sterile loop and then transferring large numbers of organisms to 50 ml LB-medium supplemented with $100 \mu\text{g}\cdot\text{ml}^{-1}$ ampicillin. Liquid cultures were incubated overnight in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., USA) at 37°C and 200 rpm (2 cm amplitude).

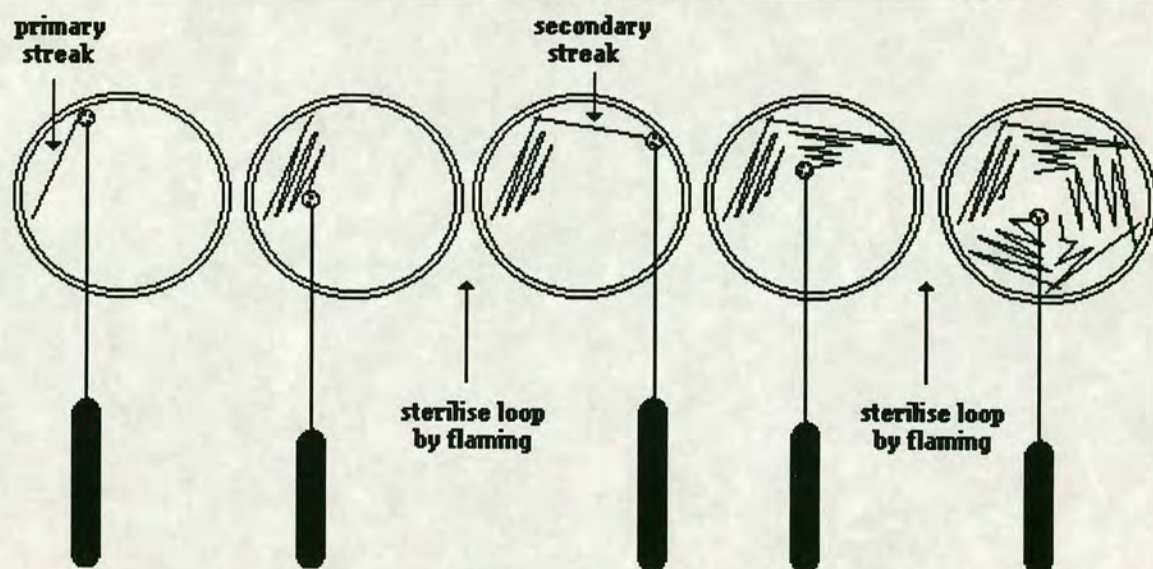


Figure 2.3.5 Streaking technique (Maniatis *et al.*, 1982). A platinum transfer loop, sterilised by flaming and cooled in sterile water, was used to pick up bacteria from a stock culture (stab culture or frozen liquid culture). The bacteria that adhere to the loop were transferred to a sterile tube containing 1 ml liquid medium. The loop was sterilised and cooled as above, dipped into the bacterial suspension and streaked onto a segment of a plate containing agar medium. The sterilised loop was passed across one end of the primary streak and bacteria were spread into a fresh region of the agar medium. The loop was sterilised again and streaked from one end of the secondary streak. The procedure was repeated until the entire surface was covered. The lid was replaced and the plate was incubated in an inverted position at 37°C overnight. Well separated colonies were visible in the area of the final streak.

Table 2.3.4 Constituents of LB (Luria-Bertani) medium used for *E. coli* cultures (Sambrook *et al.*, 1989). Solid medium contained 1.5% Bacto™ agar (Difco Laboratories, USA). 5 N NaOH was used to adjust to pH 7.0. The medium was sterilised for 20 min at 121°C (15 psi steam pressure) and cooled to 50°C (water bath) before ampicillin was added from filter sterilised stock solution (25 mg·ml⁻¹, stored in aliquots at -20°C).

Bacto™ tryptone	10.0 mg·ml ⁻¹
Bacto™ yeast extract	5.0 mg·ml ⁻¹
NaCl	10.0 mg·ml ⁻¹
Ampicillin	100.0 µg·ml ⁻¹

2.4 Elicitation of *Capsicum frutescens* cell cultures

Suspension cultures of *Capsicum frutescens* were elicited with aqueous extracts of *Gliocladium deliquescens* spores. The concentration of fungal elicitor was expressed as the concentration of detectable carbohydrate. Elicitor preparations containing 2 mg carbohydrate (equivalent 30 mg spores) were used to elicit 50 ml cell suspension for the times stated in the text. The cultures were harvested by vacuum filtration at the end of the elicitation period and the cells were washed thoroughly with dist. water. The cells were frozen in liquid nitrogen and stored at -70°C until used, except for PAL activity which was extracted from fresh material.



Figure 2.4.1 Scanning EM photograph of a single conidiophore of *Gliocladium deliquescens* Sopp.

2.4.1 Preparation of fungal elicitor

Gliocladium deliquescens cultures were grown as described in section 2.3.2. The cultures are dark green (Fig. 2.3.3 part B) due to the presence of large numbers of conidia (Figs. 2.3.4, 2.4.1, 2.4.2).

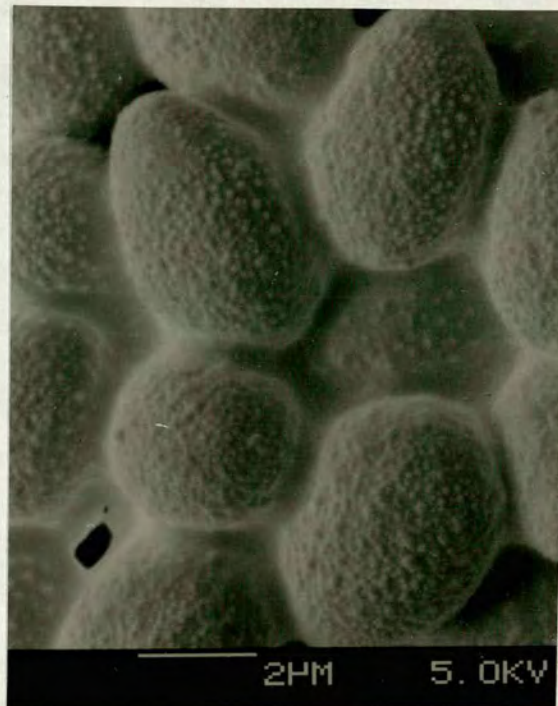


Figure 2.4.2 Scanning EM photograph of spores of *Gliocladium deliquescens* before harvesting and drying.



Figure 2.4.3 Scanning EM photograph of air dried spores of *Gliocladium deliquescens*

Approximately one week after inoculation, the surface material (mycelia and conidia) was removed with a spatula and transferred to a screw cap centrifuge tube. The combined material of several plates was vortexed thoroughly with an excess volume of abs. ethanol. The slurry was filtered through nylon mesh (200 μm pore size) to remove larger aggregates. The filtrate was air dried at 60°C and the dried spores (Fig. 2.4.3) were stored in a screw cap centrifuge tube sealed with Parafilm™ at -3°C (refrigerator ice box). Spores (30 mg·ml⁻¹) were suspended in dist. water and autoclaved at 121°C (15 psi steam pressure) for 20 min. The suspension was filtered through Rainin Nylon-66 filters (0.2 μm pore size, Anachem, U.K.) using a Sartorius filtration unit and glass fibre prefilters (Schleicher & Schuell, Germany). The filtrate was assayed for carbohydrates and used as fungal elicitor for *Capsicum* suspension cultures.

2.4.2 Carbohydrate determination

Total carbohydrate concentration of the elicitor preparation was estimated following a method of Dubois *et al.* (1956) as described by Fry (1988). Ten μl of water-saturated phenol (Rathburn, U.K.) was added to a 400 μl aqueous sample containing 2-15 μg carbohydrate in a 15 ml test tube. One ml conc. H_2SO_4 was then pipetted directly into the aqueous solution to ensure maximum mixing. After 10 min the solution was cooled in a bath of tap water for 10-20 min and the absorbance at 485 nm was measured against a blank, containing 400 μl water instead of the sample, using a Beckman DU-64 spectrophotometer. The amount of carbohydrate, expressed as glucose equivalents, was calculated using a calibration curve (Fig. 2.4.4).

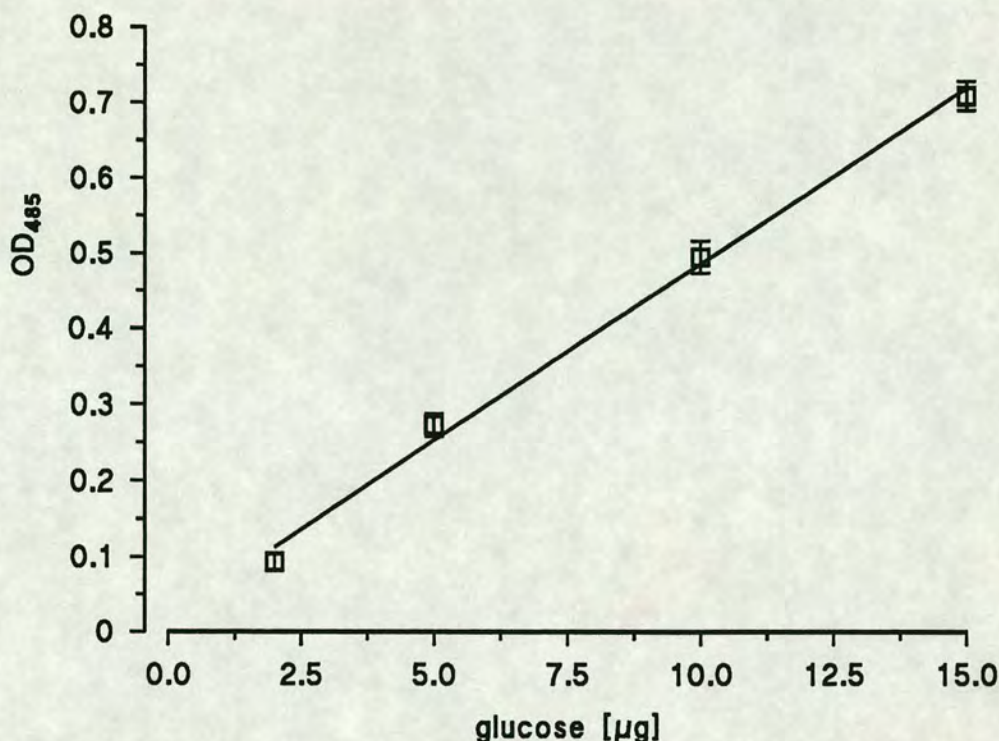


Figure 2.4.4 Calibration curves to determine the amount of carbohydrate in the elicitor preparation were plotted using D-glucose. The elicitor carbohydrate was expressed as glucose-equivalents. Each data point represents the arithmetic mean of three replicates \pm SE

2.5 Extraction and determination of PAL activity

2.5.1 Enzyme extraction

Extraction of PAL activity was based on the methods of Hahlbrock and Wellmann (1973) and Ozeki and Komamine (1985) as described by Holland (1989). The extraction was carried out at 4°C (cold room). Tissue (4 g) was ground for 1 min in a chilled mortar on ice with 0.4 g insoluble PVP (Polyclar AT, BDH, U.K.) and 12 ml extraction buffer (0.2 M Tris-HCl pH 7.8, 10 mM β -mercapto ethanol). PVP assisted in removing phenolic impurities (Loomis and Battaile, 1966). Insoluble material was pelleted at 2600xg for 20 min (4°C). The supernatant was incubated with 0.4 g Dowex 1x4 200 (Sigma), equilibrated in 4 ml extraction buffer, for 20 min on ice to remove polyphenolics. The Dowex was pelleted by centrifugation as described

above. The volume of the supernatant, forthwith referred to as crude enzyme extract, was adjusted to 20 ml and assayed for PAL activity.

2.5.2 PAL assay

PAL activity was determined using a spectrophotometric assay, the principle of which was outlined by Zucker (1965). The increase in absorbance during the reaction period was measured at 290 nm wavelength against a blank without substrate. The observed increase in optical density is proportional to the amount of cinnamic acid produced since the substrate phenylalanine contributes little absorption at this wavelength (Fig. 2.5.1). Enzyme

activity was assayed at pH 8.8, the pH optimum of *Capsicum* PAL (Holland, personal communication). 3 ml (4 ml for blank) 0.2 M Tris-HCl pH 8.8 and 300 μ l crude enzyme extract were pre-incubated at 40°C for 10 min. The enzyme reaction was started by addition of 1 ml 50 mM L-phenylalanine to give a concentration of *ca.* 12 mM in the reaction mixture. Absorbance was measured at the start of the reaction (t_0) and after 2 h reaction

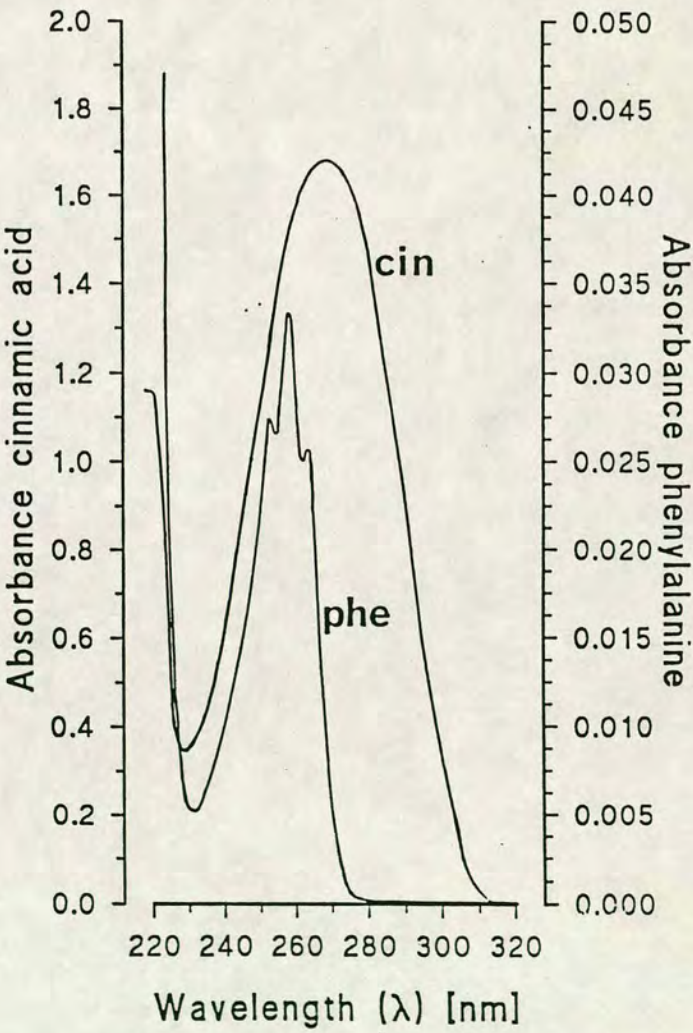


Figure 2.5.1 UV spectra of 0.1 mM *trans*-cinnamic acid and 0.1 mM L-phenylalanine. At 290 nm the absorbance of L-phenylalanine is practically zero.

time (t_{2h}) at 40°C using a Pye-Unicam SP8-100 ultraviolet spectrophotometer. Absorbance at t_0 (usually zero) was subtracted from the absorbance at t_{2h} to calculate the net increase in absorbance.

2.5.3 Calculation of the specific PAL activity

Enzyme activity was expressed as moles product per second, the unit of which is the katal (kat). The extinction coefficient for cinnamic acid (ΔE_{290}) was determined to be 10^4 [l·mol⁻¹·cm⁻¹] under assay conditions (Fig. 2.5.2). Enzyme activity was related to the total protein content of the crude enzyme extract to calculate the specific enzyme activity. The results of this were expressed as [μ kat·(kg protein)⁻¹]. Total protein content was determined as described in section 2.6.

$$[\text{kat}] = \frac{\text{OD}_{290} \times \text{react.vol.}}{\Delta E_{290} \times \text{react.time}} \quad [\text{mol} \cdot \text{sec}^{-1}]$$

$$[\mu\text{kat}] = \frac{\text{OD}_{290} \times \text{react.vol.} \times 10^6}{\Delta E_{290} \times \text{react.time} \times 10^6} \quad [\mu\text{mol} \cdot \text{sec}^{-1}]$$

$$[\mu\text{kat} \cdot (\text{kg protein})^{-1}] = \frac{\text{OD}_{290} \times \text{react.vol.} \times 10^6 \times 10^9}{\Delta E_{290} \times \text{react.time} \times \text{protein}} \quad [\mu\text{mol} \cdot \text{sec}^{-1} \cdot (\text{kg protein})^{-1}]$$

$$\text{OD}_{290} = \text{OD}_{290}(t_{2h}) - \text{OD}_{290}(t_0) \quad [\text{cm}^{-1}] \quad \Delta E_{290}(\text{CINNAMATE}) = 10^4 \quad [\text{l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}]$$

$$\text{reaction volume} = 4.3 \cdot 10^{-3} \quad [\text{l}] \quad \text{reaction time} = 7.2 \cdot 10^3 \quad [\text{sec}]$$

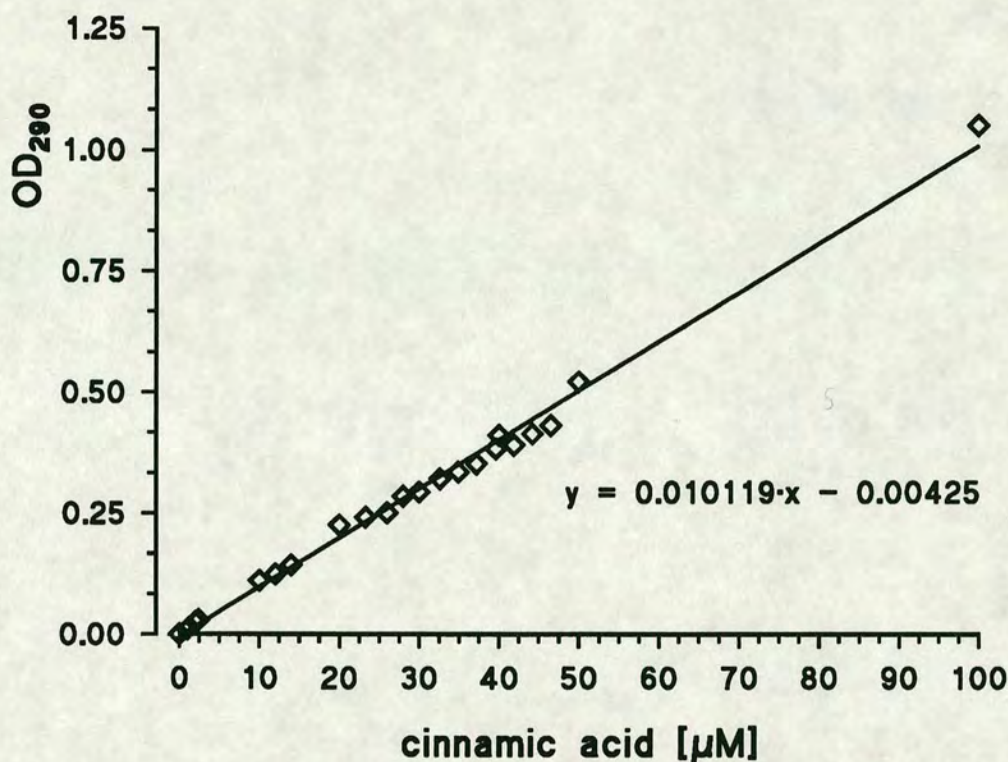


Figure 2.5.2 Estimation of $\Delta E_{290}(\text{CINNAMATE})$. The absorbance of various concentrations of *trans*-cinnamic acid was measured under assay conditions. The slope of the linear regression determines the coefficient ($\approx 10^4$).

2.6 Determination of soluble protein concentration

Protein concentration was determined following the method of Bradford (1976) except that the assay volume was reduced to 3.1 ml. The reagent was prepared by dissolving 50 mg Coomassie Brilliant Blue G-250 (Sigma) in 25 ml abs. ethanol. The solution was homogenised using a Sorvall Omnimixer to assist solubilisation. The homogenate was adjusted to 400 ml with dist. water and stirred overnight in the dark. After addition of 50 ml *ortho*-phosphoric acid the reagent was adjusted to 500 ml, vacuum filtered through 0.2 μm pore size aluminium filter (Anachem, U.K.), and stored at room temperature in the dark.

Aliquots of crude enzyme extract were adjusted to 10% TCA with 100% TCA stock solution¹ and allowed to stand at 4°C overnight to precipitate soluble protein (Lowry *et al.*, 1951). Precipitated protein was pelleted using an Eppendorf centrifuge and resuspended in 0.1 N NaOH. Samples were diluted as required and 100 µl aliquots were thoroughly mixed with 3 ml dye reagent. After 5 min the absorbance was measured at 595 nm using a Beckman DU-64 spectrophotometer against a blank consisting of 3 ml reagent and 100 µl 0.1 N NaOH. Protein concentrations were estimated using a BSA calibration curve (Fig. 2.6.1).

2.7 Radiolabelling of protein

2.7.1 *In vivo* labelling with [³⁵S]amino acids

Growing *Capsicum frutescens* suspension cultures (50 ml) were labelled with 1.85 MBq of a mixture of [³⁵S]methionine and [³⁵S]cysteine (EXPRES³⁵S³⁵S labelling mix™, DuPont, USA) for the time intervals stated in the text. The labelling mixture is an unspecific hydrolysate of *Escherichia coli* grown in the presence of carrier free ³⁵SO₄. About 77% of the label in the hydrolysate is in the form of [³⁵S]methionine, with the remainder consisting of [³⁵S]cysteine (18%) and a number of oxidised by-products. The specific activities were 419.95 MBq·ml⁻¹ total hydrolysate and 43.9 TBq·mmol⁻¹ [³⁵S]methionine. The cultures were harvested by vacuum filtration and the amount of radiolabel in the medium was determined by liquid scintillation counting (section 2.7.2). Cells were extracted as described in section 2.5.1. The protein content in the crude enzyme extract was estimated (section 2.6) and the amount of radiolabel in the enzyme extract was determined by scintillation counting. Crude enzyme extract was subjected to SDS-PAGE (section 2.8) and fluorography (section 2.11.2).

¹ 100% TCA stock solution was prepared as described by Maniatis *et al.* (1982)

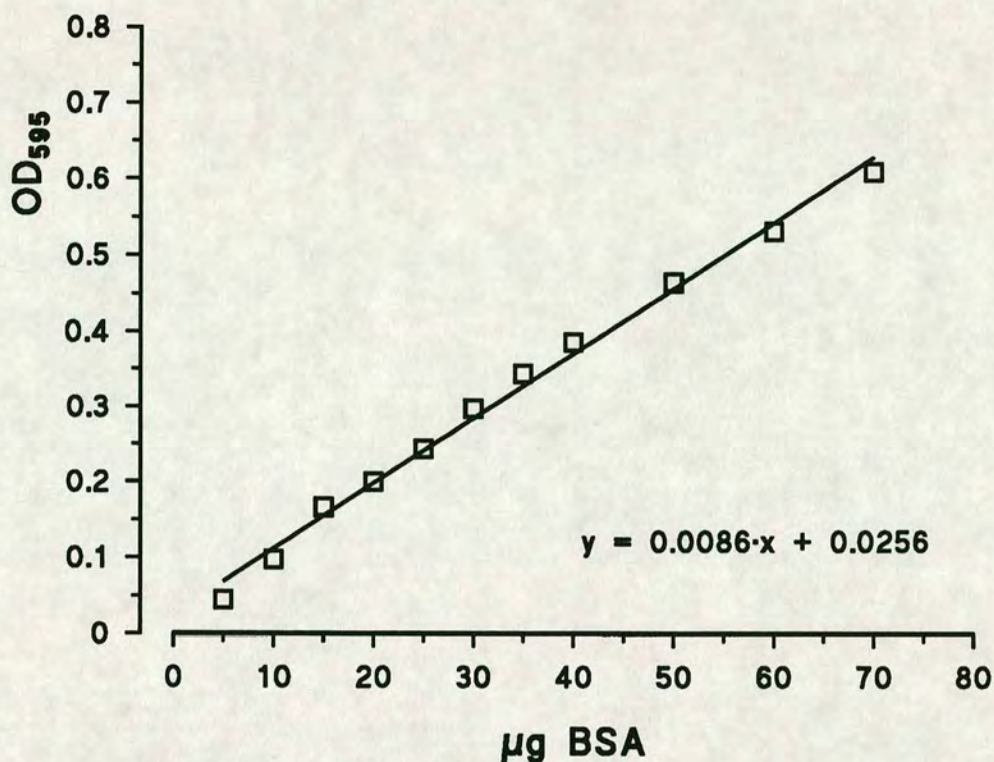


Figure 2.6.1 Protein assay (Bradford, 1976) calibration curve prepared with various amounts of BSA in 100 µl sample volume

2.7.2 Liquid scintillation counting

The scintillation cocktail consisted of 6.1 g butyl-PBD dissolved in 1 l toluene plus 500 ml Triton X-100 (BDH, U.K.) for aqueous samples. The radioactive sample (up to 1 ml) was mixed with 4 ml scintillation cocktail in a polythene vial. The sample was counted in an Intertechnique SC3000 scintillation counter. The results were obtained as counts per minute (cpm) which were then converted to disintegrations per minute (dpm) using the external standard ratio method. The quench correction curves (see Fig. 2.7.1 for [³⁵S]methionine) were obtained by counting a known activity of the relevant isotope, and sequentially quenching the sample with aliquots of acetone. Different χ -values were obtained as the amount of acetone, and therefore the quenching of the sample, increased. The percentage counting efficiency was related to

the χ -values in quench correction curves which were subsequently used to convert cpm to dpm for all samples counted.

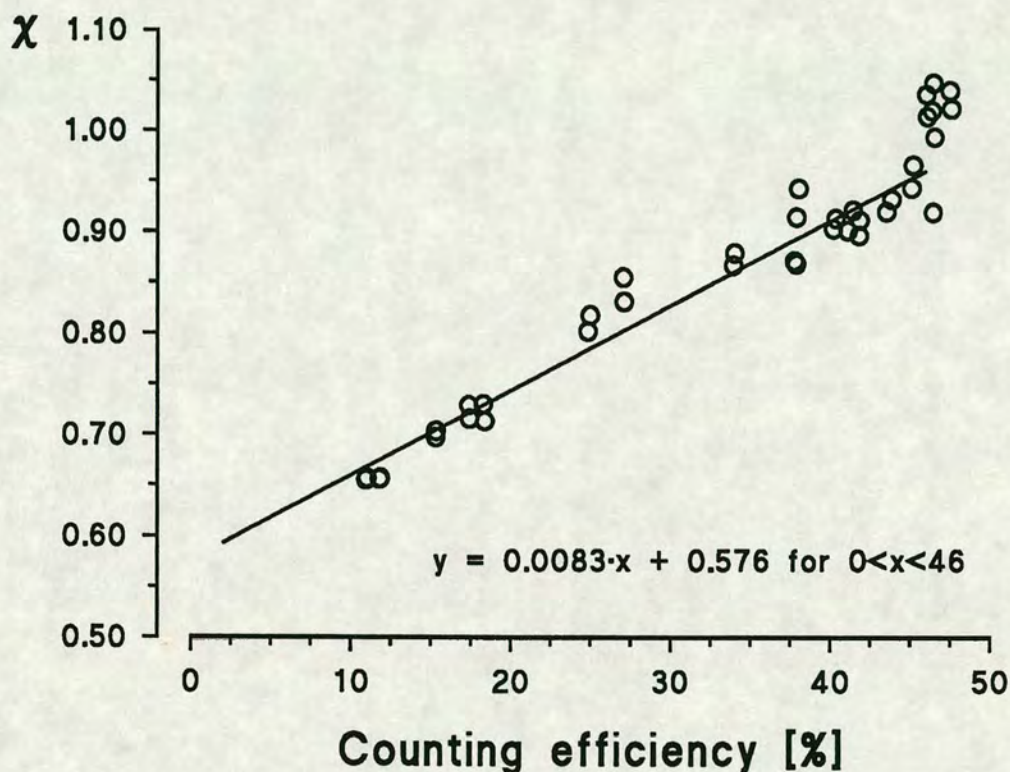


Figure 2.7.1 A quench correction curve for [^{35}S]methionine was obtained by quenching a known quantity of [^{35}S]methionine (2.5 μCi) with various amounts of acetone. The counting efficiency was plotted against the χ -value (external standard ratio method). The linear part of the curve was used to convert cpm to dpm

2.8 SDS-PAGE

Vertical SDS-PAGE was performed following the modified method of Laemmli (1970) as described by Miedzybrodzka (1980). Polyacrylamide gels were formed by co-polymerisation of acrylamide and bis-acrylamide. The reaction is a vinyl addition polymerisation initiated by a free radicle-generating system (Richards and Lecanidou, 1974). Polymerisation was initiated by TEMED and ammonium persulphate (AMPS).

The AMPS yields a persulphate free radicle which, in turn, activates TEMED. The TEMED acts as an electron carrier to activate the acrylamide monomer, providing an unpaired electron to convert the acrylamide monomer to a free radicle. The activated monomer then reacts with unactivated monomer to begin the polymer chain elongation. The elongating polymer chains are randomly crosslinked by bis, resulting in closed loops and a complex "web" polymer with a characteristic porosity which depends on the polymerisation conditions and monomer concentrations. SDS forms complexes with proteins, surrounding the protein molecule and eliminating charge differences of the proteins, therefore the separation occurs on the basis of molecular size. Addition of SDS also prevents aggregation during electrophoresis because of its capacity to split hydrogenic and hydrophobic bonds.

2.8.1 Polyacrylamide gels

Polyacrylamide gels of various pore sizes were prepared by mixing stock solutions according to Table 2.8.1. Acrylamide stock solution consisted of a mixture of 30% acrylamide and 0.2% bis-acrylamide. Tris stock solution was 1.875 M Tris-HCl (pH 8.8) for separating gels and 0.6M Tris-HCl (pH 6.8) for stacking gels respectively, resulting in different pH and ionic strength for both gel types.

10% SDS was heated to 65°C for 1 h and stored at 37°C. 10% AMPS was freshly prepared before use.

The final concentrations in the gel mixtures were as follows

Stacking gel	Separating gel
0.06 M Tris-HCl, pH 6.8	0.375 M Tris-HCl, pH 8.8
5% acrylamide	5-20% acrylamide
0.033% bis-acrylamide	0.033-0.18% bis-acrylamide
0.1% SDS	0.1% SDS
0.1%(v/v) TEMED	0.06%(v/v) TEMED
0.05% AMPS	0.05% AMPS

The gel mixtures excluding SDS and TEMED were degassed by applying vacuum for 15-20 min to exclude oxygen which would inhibit polymerisation. After addition of SDS and TEMED the separation mixture was poured into a mould (Fig. 2.8.1), which was created by two glass plates in an upright position separated by 0.75 mm thick spacers on each side, sealed with silicon tubing of the appropriate diameter, and clamped together with bulldog clips. During the polymerisation period of *ca.* 20 min the top of the separating

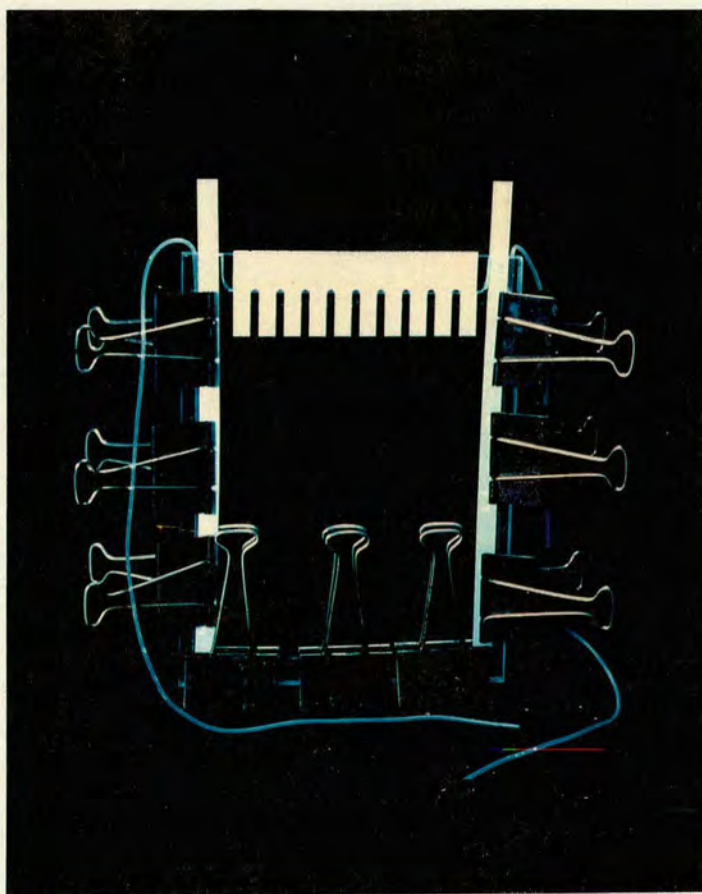


Figure 2.8.1 Polyacrylamide gels were poured into a mould created by two glass plates separated by spacers and sealed with silicon tubing. The thickness of the spacers determined the thickness of the gel.

gel was covered with a layer of water-saturated butanol. The butanol was removed with filter papers once the gel was set and topped with the degassed stacking gel mixture, into which a gel comb of the same thickness as the spacers was inserted, in order to form the sample wells. After polymerisation of the stacking gel, the silicon tubing was removed from the assembled gel unit and the unit was placed into an electrophoresis apparatus (Fig.2.8.2). Top and bottom reservoirs were filled with electrode buffer (0.05 M Tris, 0.38 M glycine, 0.1% SDS) and the gel comb was removed carefully, allowing electrode buffer to fill the wells.

Table 2.8.1 Constituents of polyacrylamide gels of different acrylamide concentrations. Gel mixtures were prepared from appropriate stock solutions by adding the stated volumes, except SDS and TEMED, which were added after degassing and immediately before pouring the gel

	separating gel					stacking gel
acrylamide concentr.	5.0 %	10.0 %	12.0 %	15.0 %	20.0 %	5.0 %
acrylamide	1.67ml	3.30ml	4.00ml	15.00ml	20.10ml	1.67 ml
Tris-HCl	2.00ml	2.00ml	2.00ml	6.00ml	6.00ml	1.00 ml
dist. H ₂ O	6.10ml	4.50ml	3.80ml	8.40ml	3.30ml	7.20 ml
10% SDS	0.10ml	0.10ml	0.10ml	0.30ml	0.30ml	0.10 ml
TEMED	10.00μl	8.00μl	6.00μl	12.00μl	7.50μl	10.00 μl
10% AMPS	50.00μl	50.00μl	50.00μl	150.00μl	150.00μl	50.00 μl
total volume	10.00ml	10.00ml	10.00ml	30.00ml	30.00ml	10.00 ml

2.8.2 Sample preparation

Protein samples (enzyme extracts and molecular weight markers) were adjusted to pH 6.8 and 1 volume 2x SDS-PAGE sample buffer was added. The final concentrations in the sample were as follows.

0.06 M Tris-HCl pH 6.8, 2% SDS,
5%(v/v) β-mercapto ethanol and 10%
sucrose

The density of the sample, due to the high sucrose concentration, enables the sample to sink to the bottom of the submerged sample wells.

Protein samples were incubated at 96°C for 10 min, then quickly cooled on ice. After addition of a trace of bromophenol blue the samples (50-100 μ l) were loaded onto the gel using a Hamilton syringe.

2.8.3 Electrophoresis conditions

The platinum electrodes in the top and bottom buffer reservoirs of the electrophoresis apparatus

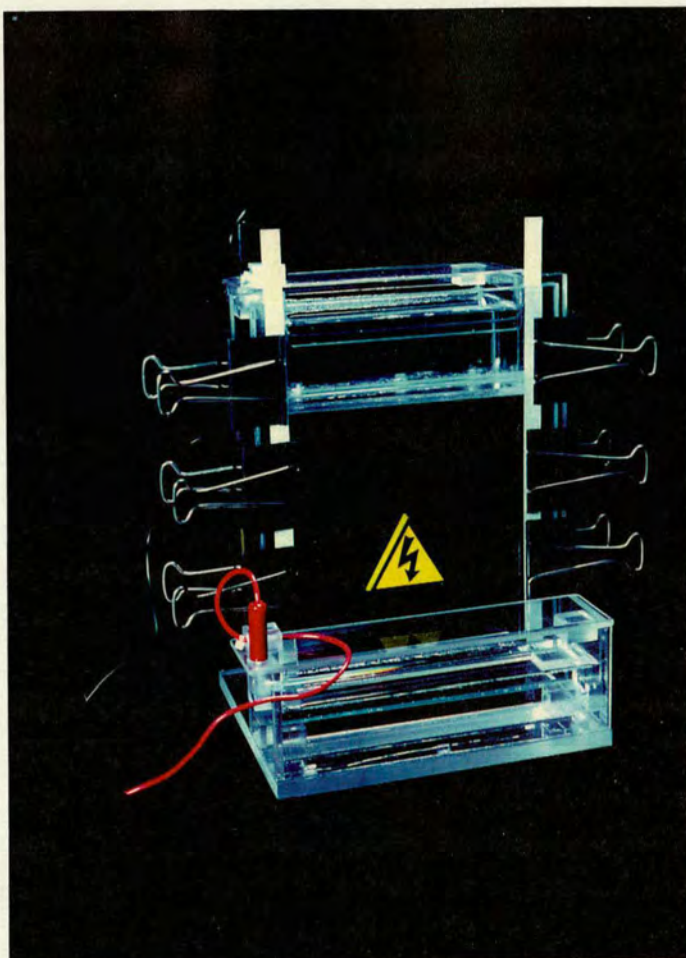


Figure 2.8.2 Electrophoresis apparatus for vertical SDS-PAGE assembled with gel unit. Electrodes in the buffer reservoirs are attached to a power pack (Top electrode to cathode, bottom electrode to anode)

were connected to a power pack (bottom reservoir to anode, top reservoir to cathode) and the proteins were separated according to their molecular size during their migration along the electrical field in the direction of the anode. Smaller proteins migrate faster than the larger molecules. Proteins of the same size migrate at the same speed. All proteins can be detected as bands upon staining.

Electrophoresis was carried out at constant current for 4.5 h (stacking gel 10 mA, separating gel 15 mA) or overnight (6 mA) until the tracker dye reached the bottom of the gel.

2.8.4 Staining of polyacrylamide gels

When the run has been completed the gel was removed from between the glass plates and, unless stated otherwise, stained with a mixture of 0.1% Coomassie Brilliant Blue in 50%(v/v) methanol and 5%(v/v) acetic acid for 1 h with constant agitation. The staining solution was stored at room temperature and re-used many times without loss of staining activity.

The gel was destained for 5 h or, when more convenient, overnight in 50%(v/v) methanol and 5%(v/v) acetic acid. The destaining solution was changed several times during the first 2 h. After destaining, the pattern of protein sub-units was visible as blue bands against a clear background. At this stage, except when fluorography was intended, the gel was dried under vacuum onto Whatman 3MM chromatography paper.

2.9 Western Blotting

The electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose, Western Blotting (Burnette, 1981; Towbin *et al.*, 1979), was performed using a Semidry Electroblotter SM 17556 (Sartorius, F.R.G.) according to the manufacturer's manual.

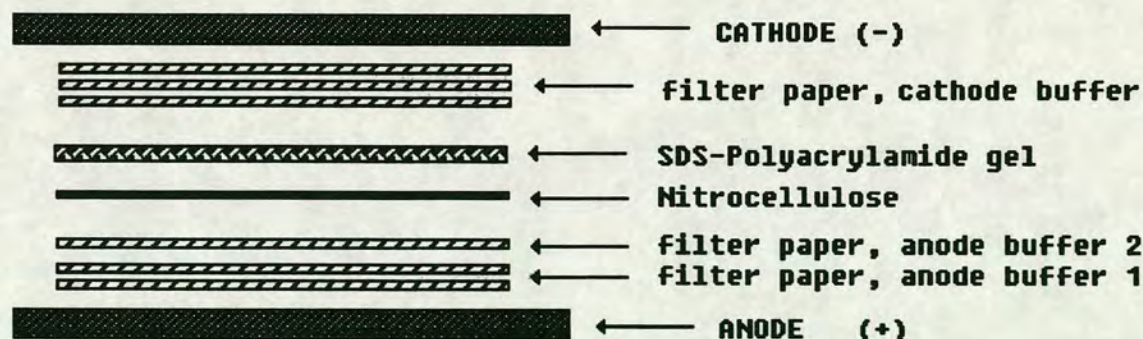


Figure 2.9.1 Transblot unit for Western Blotting. Filter papers, acrylamide gel, and nitrocellulose membrane were positioned between the graphite electrodes of the Semidry Electroblotter SM 17556 (Sartorius, F.R.G.) as shown above. Constituents of the buffers are listed in the text. Transfer was carried out at $0.8 \text{ mA} \cdot \text{cm}^{-2}$ for 1 h at room temperature.

Nitrocellulose membrane and filter papers (Whatman 3MM) were cut to the size of the polyacrylamide gel. Transblot units (Fig 2.9.1) were assembled as follows. The graphite plates (16x16 cm) were rinsed with dist. water. Two layers of filter paper soaked in anode buffer No.1 (0.3 M Tris, 20%(v/v) methanol, pH 10.4) were placed onto the anode graphite plate in the bottom of the apparatus. One layer of filter paper soaked in anode buffer No.2 (25 mM Tris, 20%(v/v) methanol, pH 10.4) was placed on the two layers of filter paper soaked in anode buffer No.1. Next, the nitrocellulose membrane was placed on the layers of filter paper and the acrylamide gel on the membrane, without trapping any air bubbles in between. Finally, three layers of filter paper soaked in cathode buffer (25 mM Tris, 40 mM 6-amino-*n*-hexanoic acid (Sigma), 20%(v/v) methanol, pH 9.4) were placed on top of the acrylamide gel. The lid of the apparatus, containing the cathodic graphite plate, was positioned on top of the stack.

The transfer of protein was carried out at $0.8 \text{ mA}\cdot\text{cm}^{-2}$ for 1 h at room temperature.

Table 2.9.1 Composition of 1x PBS-Tween 20. A 10x stock solution was prepared and diluted with the appropriate volume of dist. water before use.

1x PBS-Tween 20
4.0 mM KH_2PO_4
16.0 mM Na_2HPO_4
150.0 mM NaCl
0.02 % NaN_3
0.05 %(v/v) Tween 20
pH 7.4

After the transfer, the nitrocellulose membrane was carefully removed and washed in several changes of 1x PBS-Tween 20 (Table 2.9.1) to remove any

acrylamide which may have stuck to the membrane and to remove SDS which may interfere with subsequent procedures. The membrane was then stained in Ponceau-S (0.2% Ponceau-S (Sigma) dissolved in 3% TCA) for 10 min and washed very briefly in dist. water to remove background staining. Pink coloured protein bands were visible against a clear background. The positions of molecular weight reference proteins (Cat.No. SDS-6H, Sigma) were marked with a pencil. The membrane was then dried between two filter papers for 15 min at 80°C under vacuum. The dried membrane was stored at 4°C in a sealed polythene bag until used (up to 24 h). The polyacrylamide gel was routinely stained as described in section 2.8.4 to check the efficiency of the transfer.

2.10 Immunodetection of PAL sub-units

The transfer of protein bands to nitrocellulose sheets occurs without the loss of resolution but is not quantitative. Before the immobilised protein can be visualised using immunological techniques all unoccupied protein-binding sites on the nitrocellulose membrane must be occupied (blocked) by flooding with an excess of protein (Towbin *et al.*, 1979). Several methods and reagents are described for blocking free binding sites on nitrocellulose membranes (Bird *et al.*, 1988; Spinola and Cannon, 1985). BSA, non-fat dry milk, gelatine and Tween 20 were tested for the experimental system used. Commercially obtained non-fat dry milk in PBS-Tween 20 (Johnson *et al.*, 1984) resulted in the lowest background stain and was subsequently used for all experiments.

The dried, Ponceau-S stained, nitrocellulose membrane was wetted and unstained in PBS-Tween 20 for 30 min with constant agitation. The completely unstained blot was incubated in blocking reagent (10% non-fat dry milk in PBS-Tween 20) for 2 h. The blocked membrane was washed in PBS-Tween 20 containing 0.1% BSA for 20 min. The membrane was then hybridised with primary antibody for 2 h in a sealed

plastic bag to reduce the volume of hybridisation solution to a minimum. The hybridisation solution consisted of a 1:200 dilution of rabbit anti-(parsley PAL) serum in PBS-Tween 20 containing 0.1% BSA. The anti-serum (Anti-PAL 592/c) was a kind gift of Prof. Dr. K. Hahlbrock (MPI Cologne, F.R.G.). Unspecific hybridisation was removed by 20 min washing cycles with each of the following reagents.

- PBS-Tween 20 (twice)
- PBS-Tween 20 containing 0.5 M NaCl
- PBS-Tween 20
- PBS-Tween 20 containing 0.1% BSA

The nitrocellulose membrane was then hybridised with secondary antibody for 2 h in a sealed plastic bag. The hybridisation solution consisted of a 1:100 dilution of affinity-purified GAR antibodies labelled with colloidal gold particles (AuroProbe™ BLplus, Jansson, Belgium) in PBS-Tween 20 containing 0.1% BSA and 5% gelatine (IGSS quality, Jansson, Belgium). After the hybridisation the blot was washed for 20 min with each of the following reagents.

- PBS-Tween 20 (twice)
- PBS-Tween 20 containing 0.5 M NaCl
- PBS-Tween 20 (twice)

The hybridisation with AuroProbe™ BLplus was used to visualise the binding between primary antibody and antigen. Due to the optical characteristics of colloidal gold label (Horisberger and Clerc, 1985; Brada and Roth, 1984) the AuroProbe™ reagent produces a pinkish signal which was enhanced with silver reagent, resulting in much greater sensitivity and contrast. Sensitivity of *ca.* 1 ng is reported by Brada and Roth (1984). Enhancement was achieved by the precipitation of metallic silver on the colloidal gold particles. This resulted in a high-contrast dark brown to black signal. Photochemical silver staining of the Western Blot was performed using IntenSE™ BL (Jansson, Belgium) silver staining kit, but it was found that a cleaner background was achieved using the following method.

The Western Blot was washed twice in dist. water for 1 min to remove chloride ions. Longer periods of time in dist. water lead to a release of gold particles from the

membrane (AuroProbe™ BLplus manual). The membrane was then washed in 0.2 M citrate buffer (2.35 g trisodium citrate + 2.55 g citric acid in 100 ml volume) for 2 min. Next, the blot was incubated in silver enhancer (77 mM hydroquinone, 5.5 mM silver lactate in 0.2 M citrate buffer)² for 2-15 min in the dark. The progress of the reaction was checked by inspection under a safelight. The blot was fixed in a 10-fold dilution of Jansson fixative solution for a maximum of 5 min. Longer periods caused image fading. Finally, the membrane was washed in water and air-dried between filter papers.

2.11 Autoradiography and fluorography

2.11.1 Direct autoradiography

Hyperfilm™ MP (Amersham, U.K.) was used for direct autoradiography and fluorography. Direct autoradiography was used for [¹⁴C] labelled samples separated on TLC plates. The sample and the film were pressed in close contact between two PVC plates. These were held in place with adhesive tape and wrapped in a black plastic bag. The film was exposed to the radioactive sample for the appropriate time (2-3 weeks) at room temperature and developed using an automatic X-ray film processor.

The silver halide crystals in the emulsion respond directly to β -particles emitted from the sample. Each emission converts several silver ions to silver atoms to produce a stable latent image. When the film is subsequently developed these few silver atoms catalyse the reduction of the entire silver halide crystal (grain) to metallic silver to produce an autoradiographic image of the radioisotope distribution (Laskey, 1984).

² Solution A: 10 ml citrate buffer from the previous step and 60 ml water
Solution B: 0.85 g hydroquinone in 15 ml water
Solution C: 0.11 g silver lactate in 15 ml water
A was added to B, mixed, then C was added, and mixed thoroughly. The solutions were handled in safelight and prepared fresh before use.

2.11.2 Fluorography

The usefulness of direct autoradiography is limited for most isotopes by inefficient transfer of their emission energy to the film. First, the problem arises when low energy emissions from radioisotopes are embedded in a polyacrylamide gel (Skinner and Griswold, 1983). Second, the opposite problem limits sensitivity for highly energetic β -particles such as those from [^{32}P]. These emissions pass through and beyond the film so that only a small proportion of their energy is captured and recorded by the film, most being wasted (Laskey, 1980). The problem of internal absorption within the sample and of inefficient absorption by the film can both be overcome by converting the emitted energy to light (Laskey, 1984). The methods used to convert emissions to light make use of scintillators which emit photons of blue or ultraviolet light in response to excitation by β -particles. Thus, they represent solid state equivalents of liquid scintillation counting (Laskey, 1984). The light produced is recorded on a X-ray film. This method of scintillation autoradiography is known as fluorography (Skinner and Griswold, 1983).

Polyacrylamide gels of [^{35}S] labelled proteins were impregnated with a scintillator solution to achieve maximum contact between the isotope and the scintillator. Coomassie Blue stained gels (section 2.8.4) were washed in glacial acetic acid to remove water from the gel and then impregnated with 22% PPO in acetic acid for 1 h at 40°C. Next, the impregnated gel was incubated in dist. water for 10 min which precipitated the PPO in the gel. Precipitated PPO adhering to the surface of the gel was carefully removed and the gel was dried under vacuum onto filter paper. The dried gel was exposed to pre-flashed HyperfilmTM MP at -70°C in a light-tight metal cassette or between PVC sheets for *ca.* 24 h.

The wastage from penetration of highly energetic β -particles through and beyond the film was overcome by placing a dense, inorganic scintillator (calcium tungstate) behind the film so that emissions passing through the film were absorbed more

efficiently by the scintillator with the production of light. Fluorography with [^{32}P] labelled samples (Northern Blots) was performed using a single Cronex[®] Intensifying Screen (DuPont, USA) placed on top of pre-flashed Hyperfilm[™] MP (Amersham, U.K.) and sample. The stack was taped into a light-tight metal cassette and exposed for the appropriate time period (12-24 h) at -70°C . A single calcium tungstate intensifying screen can increase sensitivity 10-fold for [^{32}P] compared to direct autoradiography (Laskey and Mills, 1977).

2.11.2.1 Low temperature and hypersensitised film

For a silver halide crystal of the film to be developed by the developing solution it must accumulate several atoms of metallic silver (*ca.* 5 in average emulsion). A single hit by a β -particle can produce hundreds of silver atoms, but a single hit by a photon of light produces only a single silver atom. The problem is that although two or more silver atoms in a grain are stable, a single silver atom in a silver halide crystal is unstable and it reverts to a silver ion with a half-life of about 1 sec at room temperature (Laskey, 1984). Lowering the temperature to -70°C increases the half-life of a single silver atom, thus increases the time available for a second photon to arrive and therefore, increases the sensitivity for low light intensities (Luthi and Waser, 1965). However, the relationship between blackening of the film and the amount of radioactivity in the sample is not linear when the emissions have been converted to light. The pre-exposure of the film to an instantaneous flash of light increases the sensitivity of the film to light emissions from small amounts of radioactivity and produces a linear relationship between the blackening of the film and the amount of radioactivity in the sample (Laskey, 1984). A conventional electronic photographic flash unit was used to hypersensitise Hyperfilm[™] MP. Intensity of the flash and distance from the film were adjusted to increase the absorbance (OD_{540}) of the developed film to 0.15 absorbance units above that of unexposed film.

2.12 Incorporation of radiolabel into phenolic compounds

2.12.1 *In vivo* labelling with [^{14}C]phenylalanine

L-[U- ^{14}C]phenylalanine with a specific activity of $15.7 \text{ GBq}\cdot\text{mmol}^{-1}$ was obtained in a filter sterilised solution of 2%(v/v) ethanol in water (Amersham, U.K.). Aliquots (0.185 MBq) were added directly to each experimental flask under aseptic conditions. Harvesting took place at various time intervals after addition of radiolabel depending on the nature of the experiment. Cells were harvested by vacuum filtration and washed thoroughly with dist. water. Culture medium and aqueous washes were combined and immediately extracted. Cells were weighed, frozen in liquid nitrogen, and stored at -40°C until required.

2.12.2 Extraction of phenolic compounds from culture medium

Phenylpropanoids and "capsaicinoids" were extracted from the combined cell culture medium and cell washes according to the method described by Hall *et al.* (1987). Non-radioactive standards (400 μg capsaicin and 100 μg each of caffeate, cinnamate, coumarate, ferulate, vanillin, and vanillylamine) in 1 ml methanol were added to increase the extraction efficiency of the labelled compounds. The mixture was extracted four times with equal volumes of chloroform and phase separated in a separating funnel. The combined extracts were dried with 10 g anhydrous Na_2SO_4 and, after filtration (glass fibre, Whatman, U.K.), were dried *in vacuo* at 50°C . The residue was redissolved in 1 ml HPLC-grade methanol (BDH, U.K.) for analysis.

2.12.3 Extraction of phenolic compounds from cultured cells

Cultured cells were extracted after thorough homogenisation in a large volume (*ca.* 10 $\text{ml}\cdot\text{g}^{-1}$) of methanol (Hall *et al.*, 1987) in the presence of added non-radioactive carriers (400 μg capsaicin and 100 μg each of caffeate, cinnamate, coumarate, ferulate, vanillin, and vanillylamine). The homogenate was stirred under N_2 in darkness for 4 h and the supernatant was removed by filtration through glass fibre

(Whatman, U.K.). The procedure was repeated with a similar volume of fresh methanol and left to stir overnight. The combined extracts were filtered through glass fibre and evaporated *in vacuo* at 35°C to remove the methanol. The remaining aqueous fraction was re-extracted with 4x 30 ml diethyl ether. This ethereal fraction, containing the soluble phenolics, was dried on its own or, when extraction per total culture was anticipated, combined with the chloroform extract of the culture medium and dried *in vacuo* at 35°C. The residue was redissolved in 1 ml HPLC-grade methanol for analysis.

2.12.4 Analysis of phenolic compounds by TLC

The following 2D-TLC system (Hall *et al.*, 1987) was used routinely to separate the extracted phenolic compounds with a high degree of resolution.

Plastic backed Kieselgel-60 TLC plates (Merck, F.R.G.) were used with the solvents benzene:glacial acetic acid (9:2, 1st dimension) and *iso*-propanol:butanol:ammonia:water (6:2:1:1, 2nd dimension). Loaded TLC plates were eluted for *ca.* 2 h in the first solvent system and air dried in a fume cupboard for 24 h before elution in the second solvent system for *ca.* 7 h. The plates were allowed to stand overnight in a fume cupboard to evaporate the solvents. Upon drying, the plates were subjected to direct autoradiography (section 2.11.1). Next, the TLC plates were sprayed with a freshly prepared solution of 1% $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ and 0.5% $\text{K}_3\text{Fe}(\text{CN})_6$ to localise the phenolics.

2.12.5 Analysis of phenolic compounds by HPLC

A Hewlett Packard HP 1090 liquid chromatograph fitted with a binary DR 5 solvent delivering system was used in conjunction with a HP 1040 diode array detection system (Hewlett Packard, U.K.) fitted with a 4.5 μl flow cell. Separation was achieved using a 5 μm Spherisorb C8 (200x5 mm) column (Phase Separations, U.K.) at 40°C with a varying mixture of HPLC-grade methanol and 5%(v/v) acetic acid as eluting

solvent (Hall *et al.*, 1987). All solvents used for HPLC were filtered (Rainin Nylon-66 filters, 0.2 μm pore size, Anachem, U.K.) and degassed before use. Degassing, by gently bubbling helium through the solvent with a sparger, for 10-15 min helped to prevent the formation of air bubbles within the flow cell. All samples were filtered using a microfilter (Bioanalytical Systems, U.K.) fitted with a Rainin Nylon-66 membrane filter (Anachem, U.K.) by centrifugation at 1000 \times g for 5 min. The filtered samples were stored in 2 ml autosampler vials (Chromacol, U.K.) and sealed with a crimp top. Samples were spiked with non-radioactive standards (caffeate, capsaicin, cinnamate, coumarate, ferulate, vanillin, and vanillylamine) which were detected as peaks due to their absorbance at 280 nm and identified by their characteristic spectra between 240 and 352 nm (Fig. 2.12.1). A programmable LKB 2211 Superrac fraction collector (LKB, Croydon, U.K.) was used to collect fractions as they were eluted from the Hewlett Packard flow cell. A delay of 13 sec was included in the programme to account for the time between UV detection of a compound and its subsequent collection. Fractions were analysed for radioactivity using liquid scintillation counting as described in section 2.7.2.

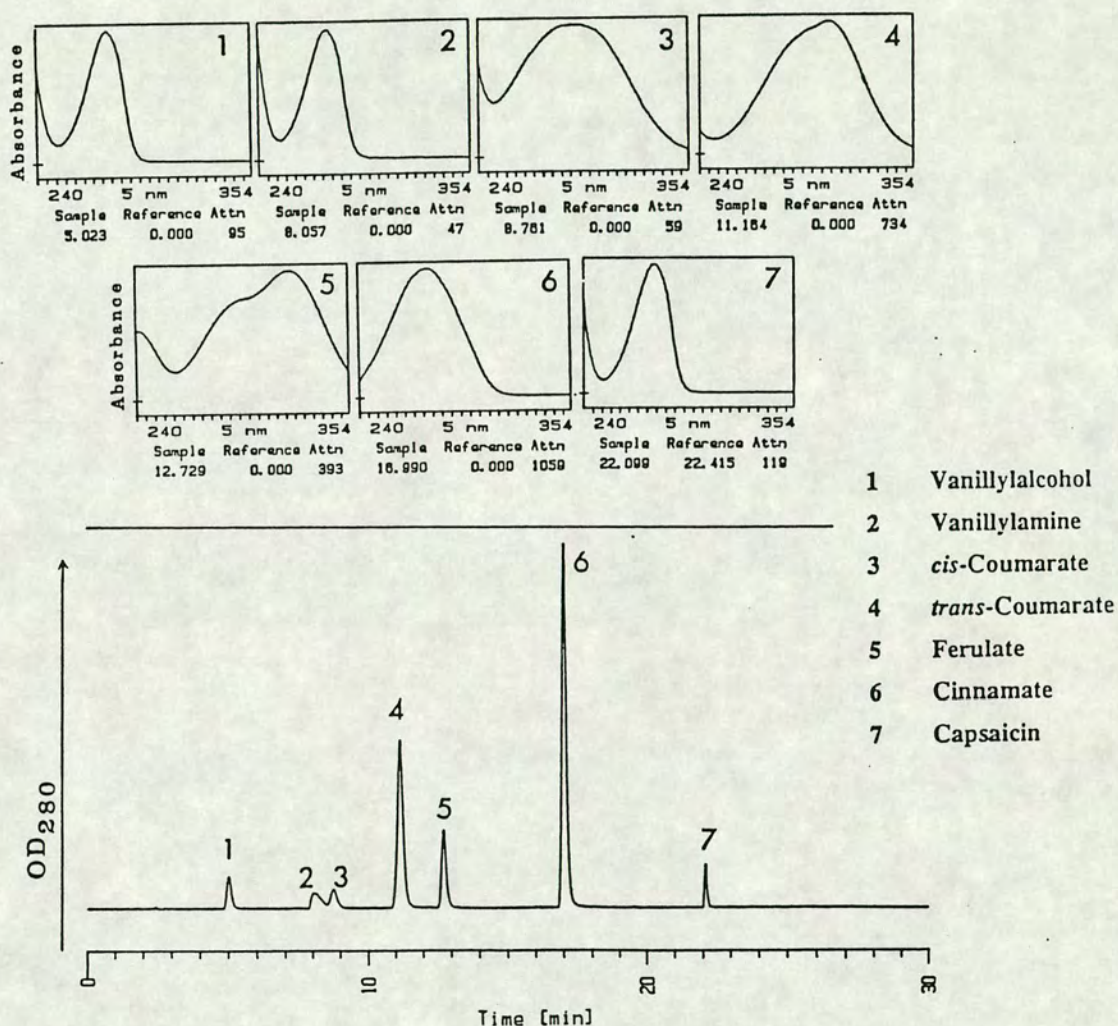


Figure 2.12.1 HPLC trace of a standard solution of phenolic compounds (capsaicin, cinnamate, coumarate, ferulate, vanillyl alcohol, and vanillylamine). The UV spectra (240-352 nm) of the eluted compounds are drawn above and the compounds are identified on the chromatograph.

2.13 Laboratory procedures for controlling ribonuclease activity

In order to obtain good preparations of eucaryotic mRNA, it is necessary to minimise the activity of RNase liberated during cell lysis (Chirgwin *et al.*, 1979) by using methods that disrupt cells and inactivate RNases simultaneously (section 2.15). Consequently, it is also important to avoid accidental introduction of trace amounts of RNase from other potential sources in the laboratory (Sambrook *et al.*, 1989).

2.13.1 Treatments for glassware, plasticware, and electrophoresis tanks

General laboratory glassware is often a source of RNase contamination (Maniatis *et al.*, 1982). All glassware, spatulas, and magnetic stirrer bars used for experiments involving nucleic acids was treated by baking at 180°C overnight. Commercially obtained non-sterile, disposable plasticware (e.g. Eppendorf tubes and pipette tips) was autoclaved before use. Sterile, disposable plasticware was used without pretreatment, since it is essentially free of RNase (Sambrook *et al.*, 1989). Gloves were worn at all stages during the preparation of materials and solutions used for the isolation and analysis of nucleic acids and during all manipulations involving nucleic acids. Electrophoresis tanks were cleaned with detergent solution, rinsed in water, dried with ethanol, and then filled with a solution of 3% H₂O₂. After 10 min at room temperature, the electrophoresis tanks were rinsed thoroughly with water that had been treated with 0.1%(v/v) DEPC (section 2.13.2).

2.13.2 Treatments for buffers and solvents

All solutions were prepared using RNase free glassware, autoclaved water, and chemicals reserved for work with RNA that were handled with baked spatulas. Wherever possible³, the solutions were treated with 0.1%(v/v) DEPC for 24 h at 37°C

³ DEPC reacts with amines and cannot be used to treat solutions containing buffers such as Tris (Sambrook *et al.*, 1989). A fresh, unopened bottle of Tris crystals was reserved for preparations of RNase free solutions which were made up with DEPC treated, autoclaved water.

and then autoclaved for 20 min at 121°C (15 psi steam pressure) where DEPC decomposes into ethanol and carbon dioxide.

2.14 Siliconisation of glassware, plasticware, and glass wool

Procedures commonly used in molecular biology can be carried out in nuclease free glassware and plasticware without significant loss of material by adsorption onto the surfaces of the containers (Sambrook *et al.*, 1989). However, for handling very small quantities of single-stranded DNA or RNA it is advisable to use glass and plastic ware that has been coated with a thin film of silicone. The following method (Sambrook *et al.*, 1989) was used for siliconising small items such as Pasteur pipettes, Eppendorf tubes, beakers, pipette tips, and glass wool.

The items to be siliconised were placed inside a large glass desiccator. 1 ml of dichlorodimethylsilane (Sigma) was added to a small beaker inside the desiccator. The valve of the desiccator was attached, through a trap, to a vacuum pump. The vacuum was turned on and suction was applied until the dichlorodimethylsilane began to boil. Immediately the desiccator's valve was closed and the vacuum pump was switched off. Great care was taken that none of the volatile agent was sucked into the pump, where it would cause irreparable damage to the vacuum seals. When the dichlorodimethylsilane had evaporated (1-2 h) the vacuum was released in a fume cupboard. After the fumes of the reagent had dispersed, the items were removed. Glassware and glass wool was baked at 180°C overnight before use. Plasticware was rinsed extensively with water and autoclaved before use.

Larger items of glassware were siliconised by rinsing in a 5%(v/v) solution of dichlorodimethylsilane in chloroform (Maniatis *et al.*, 1982). As the solvent evaporated, the dichlorodimethylsilane was deposited on the glassware, which was baked at 180°C overnight before use.

2.15 Extraction of total RNA

Frozen cells of *Capsicum frutescens* were ground in a chilled mortar with two volumes of freshly prepared RNA extraction buffer (0.1 M Tris-HCl pH 8.5, 6% PAS, 1% TNS (Gomez Lim, 1986)). The homogenate was transferred to a Sorvall Omnimix homogenising cylinder containing two volumes of phenol mixture⁴ (phenol:*m*-cresol:8-hydroxyquinoline, 90:10:0.1) and further homogenised for three 30 sec bursts at full speed. The homogenate was filtered through four layers of autoclaved muslin into a centrifuge tube. The two phases were separated at 2600xg for 10 min. The aqueous phase was extracted with one volume of phenol-chloroform mixture (phenol mixture:chloroform:*iso*-amylalcohol, 25:24:1) and the organic phase was re-extracted with 0.5 volume RNA extraction buffer. Phases were separated as described above and the pooled aqueous phases were extracted twice with an equal volume of chloroform mixture (chloroform:*iso*-amylalcohol, 24:1).

Phenol denatures proteins and precipitates them out of the aqueous phase. However, nucleic acids have an affinity for phenol, especially A-T rich strands (Brawerman *et al.*, 1972). Low temperature brings nucleic acids back to the aqueous phase. The addition of *m*-cresol to the phenol phase increases the protein denaturing effect and depresses the melting point of phenol which makes working at low temperatures possible. 8-hydroxyquinoline is an antioxidant, a partial inhibitor of RNase, and a weak chelator of divalent cations (Kirby *et al.*, 1956) and therefore suppresses the synthesis of metal catalysed oxidation products, nucleic acid-protein binding, and nuclease activity. In addition, its yellow colour provides a convenient way of identifying the organic phase. Extraction with phenol:chloroform takes advantage of the fact, that deproteinisation is more efficient when two different organic solvents are used instead of one. The subsequent extraction with chloroform removes any traces of phenol from the nucleic acid preparation.

⁴ Phenol crystals were heated until dissolved, then the other constituents were added. The mixture was saturated with 0.1 M Tris-HCl (pH 7.5) and stored in a dark glass bottle at 4°C.

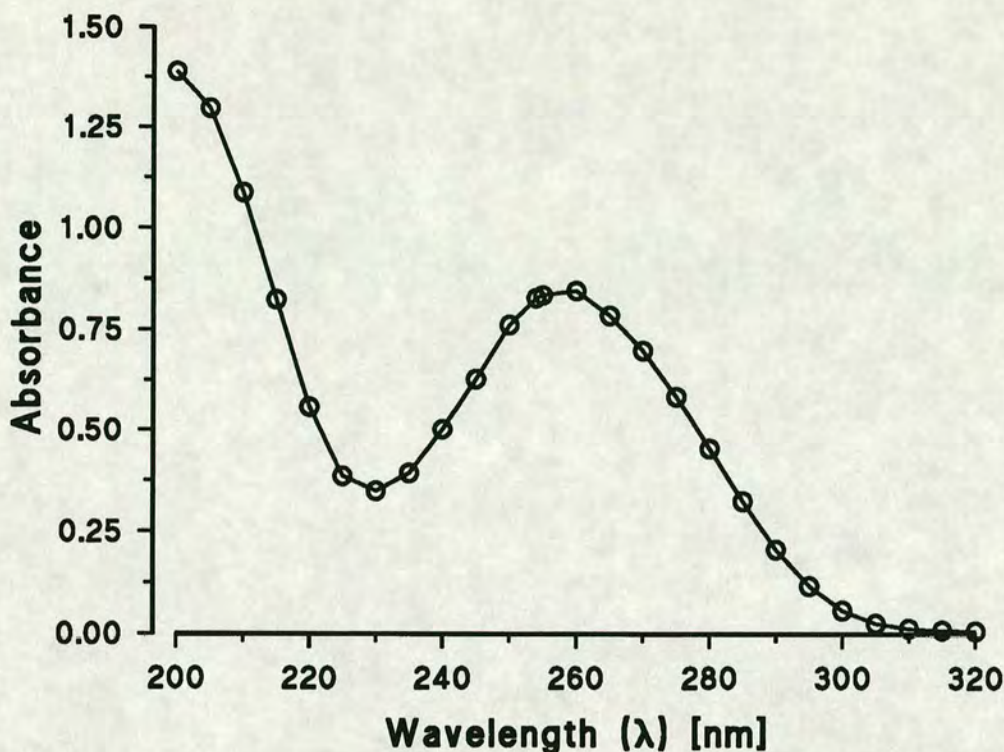


Figure 2.15.1 Wavelength scan of isolated total RNA. The relevant spectrum is between 230 and 320 nm. An optical density of 1.0 at 260 nm (OD_{260} , 1 cm path) corresponds to $40 \mu\text{g}\cdot\text{ml}^{-1}$ single stranded RNA (Maniatis *et al.*, 1982). The ratios OD_{260}/OD_{280} and OD_{260}/OD_{230} provide an estimate for the purity of the RNA preparations. Pure preparations have ratios of 2.0 and an OD_{320} that is not more than a few percent of the OD_{260} . If there is a contamination with protein or phenol, the OD_{260}/OD_{280} is significantly less than 2.0, and accurate quantification is not possible. Phenol has a strong UV absorbance and shifts the maximum to 270 nm (Maniatis *et al.*, 1982). An OD_{260}/OD_{230} less than 2.0 indicates contamination with polysaccharides (Schleif and Wensink, 1981). The extraction method described in the text routinely yielded RNA preparations of high purity.

The aqueous phase was adjusted to 0.3 M sodium acetate with a 3 M stock solution (pH 5.2) and nucleic acids were precipitated with 3 volumes of cold (-20°C) abs. ethanol at -20°C for 2 h or at 4°C overnight. RNA tends to stay in solution during ethanol precipitation which was prevented by the elevated salt concentration in the aqueous part.

The precipitate was pelleted for 30 min at 12000xg (4°C) and washed twice with 70%(v/v) ethanol to remove the salt. The pellet was washed once with abs. ethanol, dried under a stream of nitrogen, and dissolved in double dist. water. The nucleic acid solution was adjusted to 3 M sodium acetate with a 4 M stock solution (pH 5.2) and total RNA was precipitated overnight at *ca.* -3°C (refrigerator ice box). Concentrated solutions of electrolytes (1-3 M NaCl, 1-3 M LiCl, 2-3 M CH₃COONa) precipitate high molecular weight RNA, whereas DNA and low molecular weight RNA stay in solution (Wobus *et al.*, 1980).

RNA was pelleted for 30 min at 12000xg (4°C). The pellet was washed once with 3 M sodium acetate, twice with 70%(v/v) ethanol, and once with abs. ethanol. Pelleted RNA was drained carefully, but not dried out. RNA was dissolved in 300 µl double dist. water and the solution was transferred to an Eppendorf tube. A small aliquot was diluted to 1 ml and scanned from 200 to 320 nm using a Pye Unicam SP8-100 ultraviolet spectrophotometer to estimate the total amount and the purity of the extracted RNA (Fig. 2.15.1). The remaining RNA solution was adjusted to 0.3 M sodium acetate and RNA was precipitated with 900 µl cold (-20°C) abs. ethanol at -20°C for 2 h. The precipitate was pelleted for 15 min in a microcentrifuge (4°C). The pellet was washed twice with 70%(v/v) ethanol, once with abs. ethanol and dried briefly under a stream of nitrogen. The RNA was dissolved in double dist. water to a concentration of 4-4.5 µg·µl⁻¹.

2.16 Isolation of poly(A)+RNA

Polyadenylated RNA (poly(A)+RNA) was separated from the bulk of total RNA by affinity chromatography on oligo(dT)-cellulose following standard procedures (Maniatis *et al.*, 1982).

Oligo(dT)-cellulose (Pharmacia) was equilibrated in binding buffer (20 mM Tris-HCl pH 7.6, 0.5 M LiCl, 1 mM EDTA, 0.1% SDS) and a 1 ml column was poured in a

siliconised (section 2.14) disposable syringe which was plugged with siliconised glass wool. The column was washed with three volumes of each of the following.

dist. water
0.1 M NaOH, 5 mM EDTA
dist. water

The pH of the column eluent was checked to be less than 8.0, then the column was washed with 5 volumes of binding buffer.

Total RNA ($4\text{--}4.5\ \mu\text{g}\cdot\mu\text{l}^{-1}$) was denatured for 5 min at 65°C . An equal volume of 2x binding buffer was added, the sample was cooled to room temperature and applied to the column. The flow-through was collected, heated to 65°C , cooled, and reapplied to the column. The column was washed with 5-10 volumes of binding buffer and 4 volumes of washing buffer (20 mM Tris-HCl pH 7.6, 0.1 M LiCl, 1 mM EDTA, 0.1% SDS). Fractions were collected and the absorbance at 260 nm was recorded. Bound poly(A)⁺RNA was eluted with 2-3 column volumes of elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.05% SDS). Eluted poly(A)⁺RNA fractions were pooled and the LiCl concentration was adjusted to 0.5 M. Next, the pooled fractions were heated to 65°C as above and applied to a regenerated column for a second cycle. Fractions containing poly(A)⁺RNA from the second cycle were pooled, adjusted to 0.3 M sodium acetate, and RNA was precipitated with 2.2 volumes of cold abs. ethanol at -70°C .

2.17 *In vitro* translation

For *in vitro* translation experiments the rabbit reticulocyte lysate cell free translation system (Amersham, U.K., prepared according to Pelham and Jackson, 1976) was used following the manufacturer's instructions, except that the reaction mixture was reduced to 14 μl . L-[^{35}S]methionine (Amersham, U.K.) with a specific activity of $41.96\ \text{TBq}\cdot\text{mmol}^{-1}$ was used as labelled amino acid in the reaction. The

constituents of the incubation mixture were pipetted in the stated order into a pre-cooled Eppendorf tube.

10.0 μ l rabbit reticulocyte lysate
2.0 μ l [35 S]methionine ($\approx 20 \mu$ Ci)
2.0 μ l RNA sample

The mixture was incubated for 1 h at 30°C. An aliquot of the reaction mixture was subjected to SDS-PAGE (section 2.8) and fluorography (section 2.11.2).

The optimum incubation time for the rabbit reticulocyte lysate system was determined in a time course experiment with TMV RNA.

Translation mixture (1 μ l) was dried onto Whatman GF/C glass fibre filter discs for estimation of total activity. During the incubation period aliquot (1 μ l) samples were taken every 5 min and added to 0.5 ml 1 M NaOH containing 5% H_2O_2 in 15 ml test tubes. When all the samples were taken they were incubated at 37°C for 10 min to hydrolyse aminoacyl-tRNA complexes. Next, 2 ml ice-cold TCA solution (25% TCA, 2% casein hydrolysate) was added and left on ice for 30 min. The mixture was filtered through glass fibre and the filter discs were washed with 8% TCA to remove unreacted [35 S]methionine. The dried filter discs were subjected to liquid scintillation counting (section 2.7.2) and the incorporation rate was estimated.

2.18 Agarose gel electrophoresis

Agarose, which is extracted from seaweed, is the unbranched galactan chain component (1,4- β -linked D-galactose and 3,6-anhydro L-galactose) of agar, the other being agarpectin. Agarose gels were cast by melting agarose (Type II Medium EEO, Sigma) in the presence of the desired buffer until a clear, transparent solution was achieved. The melted solution was then poured into a mould and allowed to harden. Upon hardening the agarose forms a matrix, the density of which is determined by the concentration of the agarose (Boffey, 1983). When an electric field is applied across

the gel, nucleic acids, which are negatively charged at neutral pH, migrate toward the anode.

2.18.1 Non-denaturing agarose gels

Electrophoresis of native double-stranded DNA was performed using the TBE buffer system (0.089 M Tris-borate, 0.01 M EDTA pH 8.0) and 0.7% agarose (Type II medium EEO, Sigma). Agarose was dissolved in a microwave oven and ethidium bromide ($0.5 \mu\text{l}\cdot\text{ml}^{-1}$) was added upon cooling to 60°C . The gel (0.5 mm thickness) was cast into a template made of a perspex plate with edges sealed using autoclave tape. A gel comb was positioned *ca.* 1 mm above the plate so that complete wells were formed after the agarose had set (≈ 45 min at room temperature). The autoclave tape and the comb were carefully removed and the gel was mounted in the electrophoresis tank in a horizontal position. TBE buffer was added to cover the gel to a depth of approximately 1 mm. The DNA sample was mixed with 0.25 volume gel loading buffer (10 mM EDTA, 40% sucrose, 0.25% bromophenol blue and the mixture was slowly loaded into the slots of the submerged gel using a micropipette. Samples containing restriction fragments of λ -DNA were used as molecular size markers.

The gel loading buffer served three purposes. 1) It increased the density of the sample, ensuring that the DNA sinks evenly into the well. 2) It added colour to the sample, thereby simplifying the loading process. 3) It contained a dye that, in an electric field, moves towards the anode at a predictable rate. Bromophenol blue migrates through agarose gels run in TBE at approximately the same rate as linear double-stranded DNA 300 bp in length (Sambrook *et al.*, 1989).

Electrophoresis was carried out at constant voltage until the tracker dye reached the bottom of the gel. The presence of ethidium bromide allowed the gel to be examined by ultraviolet illumination at any stage during electrophoresis. It contains a planar group that intercalates between the stacked bases of DNA. The fixed position

of this group and its close proximity to the bases causes dye bound to DNA to display an increased fluorescent yield compared to dye in free solution. Absorbed UV irradiation is emitted at 590 nm in the red-orange region of the visible spectrum (Maniatis *et al.*, 1982).

2.18.2 Denaturing agarose gels containing formaldehyde

Denaturing agarose gels (100 ml) for RNA electrophoresis were prepared by dissolving 1.3 g agarose (Type II medium EEO, Sigma) in 72 ml double dist. water. After cooling to 60°C, 10 ml 10x MOPS buffer (Table 2.18.1) and 17.3 ml formaldehyde solution (37%, BDH) were mixed to give final concentrations of 1x MOPS and 2.2 M formaldehyde, respectively. The gel was cast as described in section 2.18.1. Upon cooling, the gel was mounted in the electrophoresis tank and submerged in 1x MOPS. RNA samples were mixed with an equal volume of formamide sample buffer (Table 2.18.2), heated to 60°C for 5 min to denature the RNA, and snap-cooled on ice. The samples were centrifuged for a few seconds to deposit all fluid in the bottom of the Eppendorf tubes. 0.25 volume of FDE (Table 2.18.3) was added and the samples were loaded onto the gel as described in section 2.18.1. Electrophoresis was carried out for 3 h at 100 V. Samples of *Escherichia coli* RNA were used as molecular size markers.

Table 2.18.1 Constituents of 10x MOPS buffer. The solution cannot be autoclaved, but was made up with sterile water. The pH was adjusted with 5 M NaOH and the buffer was stored in darkness at 4°C.

2 M MOPS
0.5 M NaCH ₃ COO
10 mM Na ₂ EDTA

Table 2.18.2 Constituents of formamide sample buffer. Formamide (100 ml) was deionised by stirring it with 5 g of AG 501 X8(D) Mixed Bed Resin (BioRad) for 30 min at room temperature. The resin was removed by filtering.

100 µl 10x MOPS
200 µl formamide
120 µl formaldehyde

Table 2.18.3 The constituents of FDE (ficoll-dye-EDTA) were made up to 1 ml in a graduated Eppendorf tube.

0.2 ml of 0.5 M EDTA pH 8.0
0.3 g ficoll type 400 (Sigma)

After electrophoresis was completed the gel was removed from the electrophoresis tank and washed briefly with three changes of dist. water and once for 30 min with

0.1 M ammonium acetate. Next, the gel was stained for 30 min with 0.1 M ammonium acetate containing $0.5 \mu\text{l}\cdot\text{ml}^{-1}$ ethidium bromide and destained for 30 min with 0.1 M ammonium acetate.

2.19 Northern Blotting

2.19.1 Transfer of RNA from agarose gels to nylon membrane

Northern Blotting, the transfer of size fractionated RNA from electrophoretic gels to a supportive membrane (Alwine *et al.*, 1977; Thomas, 1980) and the hybridisation with DNA probes, was performed using GeneScreen^{Plus}TM Hybridisation Transfer Membrane (DuPont, USA) following the manufacturer's protocol⁵.

RNA was extracted as described in section 2.15 and size fractionated by denaturing agarose gel electrophoresis (section 2.18.2). The agarose gel was briefly rinsed in dist. water and then incubated in 30 mM NaOH, 10 mM NaCl for 30 min. Alkaline hydrolysis helped facilitate the transfer of larger RNA (>16S, Alwine *et al.*, 1979). The gel was neutralised in excess 0.1 M Tris-HCl pH 7.0 for 30 min. The gel was then soaked in 10x SSC (1.5 M sodium chloride, 0.15 M sodium citrate) for 1 h.

GeneScreen^{Plus}TM was cut to the exact size of the gel and pencil marked on the concave side of the dry membrane. The nylon membrane was wetted with dist. water and placed onto the surface of a 10x SSC solution. The membrane was soaked in 10x SSC for *ca.* 15 min.

⁵ Method of use GeneScreen^{Plus}TM : U.S. patent 4, 455, 370

RNA was transferred to GeneScreen^{Plus}TM by capillary blotting in a setup similar to the original method for transfer of DNA as described by Southern (1975). Vacuum transfer using the MilliBlotTM-V Transfer System (Millipore, U.K.) proved unsatisfactory for the transfer of large RNA fragments.

Two pieces of filter paper (Whatman 3MM), pre-wetted in 10x SSC, were placed over an elevated (over a tray filled with 10x SSC) Perspex plate so that the ends formed wicks. The agarose gel was placed on the filter paper and the soaked membrane was carefully placed on the gel so that the pencil marked side was in contact with the gel. Any air bubbles trapped between the gel and the membrane were eased out with a gloved fingertip. Six sheets of dry filter paper (cut to the size of the gel) were placed on top of the membrane. A three inch stack of absorbant paper towels was placed on top of the dry filter papers. A small glass plate and a 1 kg weight were placed on top of the stack. The transfer was allowed to continue for 16-24 h. During that time the paper towels were changed frequently and more 10x SSC was added as needed. After the transfer was completed the paper towels and the filter papers were carefully removed and the membrane was lifted away from the gel. GeneScreen^{Plus}TM was rinsed in 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate) to remove residual agarose. Membrane and gel were inspected under UV illumination for the efficiency of the transfer. The membrane with the transferred RNA facing up was allowed to dry at room temperature. Upon drying the membrane was baked for 2 h at 80°C under vacuum to reverse the formaldehyde reaction (GeneScreen^{Plus}TM protocol, DuPont). The membrane assumed its characteristic curl upon drying and was placed into a polythene bag. It was either used immediately for cDNA hybridisation or sealed into the bag and stored at 4°C for up to 24 h.

2.19.2 Hybridisation of RNA

The nylon membrane was prehybridised by treating it in 10 ml of the following solution: 1% SDS, 1 M NaCl, and 10% dextran sulphate (Table 2.19.1). The solution was added to the plastic bag containing the membrane. The bag was sealed and incubated with constant agitation for 15-30 min at 60°C. After that period of time the prehybridisation buffer was removed, denatured⁶ hsDNA (up to 150 µg·ml⁻¹) and denatured⁶ radioactive probe (10 ng·ml⁻¹) was added. The hybridisation solution was returned to the polythene bag. The resealed plastic bag was incubated with constant agitation for *ca.* 16 h at 60°C. After hybridisation the membrane was removed from the hybridisation solution and washed as follows.

- a) twice in 100 ml of 2x SSC⁷ at room temperature for 5 min
- b) twice in 200 ml of 2x SSC⁷ containing 1% SDS at 60°C for 30 min
- c) twice in 100 ml of 0.1x SSC⁸ for 30 min at room temperature

The membrane was checked for radiolabel with a Geiger counter after each washing step. The membrane was air dried on a piece of filterpaper and subjected to fluorography as described in section 2.11.2.

Table 2.19.1 Prehybridisation buffer was prepared from stock solutions (10% SDS and 50% dextran sulphate).

7 ml dist. water
2 ml 50% dextran sulphate
1 ml 10% SDS

The solution was mixed by inversion and placed in a 65°C water bath for 10-15 min. 0.58 g NaCl was added and mixed by inversion. The tube was placed back into the water bath for 15 min and then used for prehybridisation.

⁶ DNA was denatured in water by heating at 90-100°C for 10 min.

⁷ 2x SSC: 0.3 M sodium chloride, 0.03 M sodium citrate

⁸ 0.1x SSC: 0.015 M sodium chloride, 0.0015 M sodium citrate

2.20 Preparation of cDNA probes

The plasmid (pcPAL 35B) containing the (parsley) PAL cDNA was amplified in *Escherichia coli* and isolated by the alkaline extraction method (Birnboim, 1983). PAL cDNA was excised from the plasmid with restriction endonuclease *EcoRI* (Amersham, U.K.). The cDNA obtained was labelled with α -[^{32}P]CTP by using the random prime labelling technique (Feinberg and Vogelstein, 1983; 1984).

2.20.1 Plasmid preparation

Single colonies of *Escherichia coli* strain MC 1061 containing the plasmid pcPAL 35B were obtained by streaking (Fig. 2.3.5) bacteria on LB-agar (Table 2.3.4) supplemented with $100\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ ampicillin and incubation at 37°C overnight. A single colony was inoculated in 50 ml LB medium containing ampicillin and grown overnight as described in section 2.3.3. The cell suspension was transferred to a screw cap centrifuge tube and spun for 15 min at $2600\times g$. The supernatant was discarded and the pellet (cells) was resuspended in 5 ml cold (0°C) lysis buffer⁹ by vigorous vortexing and incubated on ice for 10 min. 10 ml 0.2 N NaOH containing 1% SDS was added and mixed by inverting the tube rapidly to make sure that the entire surface of the tube came in contact with the solution. The mixture was kept on ice for a further 10 min. Next, 7.5 ml potassium acetate buffer¹⁰ was added and mixed gently by inverting the tube. After another 10 min on ice, the plasmid preparation was centrifuged for 30 min at $2600\times g$. The aqueous phase (plasmids) was removed with a wide bore pipette and filtered through MiraclothTM (Calbiochem, USA) into a centrifuge tube. The filtrate was mixed with 10 ml *iso*-propanol and left at -70°C for 30 min. The mixture was then centrifuged for 5 min at $2600\times g$ which resulted in a white DNA pellet. The supernatant was discarded and the pellet was drained by

⁹ 10 mM CDTA, 50 mM glucose, 25 mM Tris-HCl pH 8.0. $2\text{ mg}\cdot\text{ml}^{-1}$ lysozyme (Sigma) was added before use.

¹⁰ Potassium acetate buffer was prepared by adding 60 ml of 5 M potassium acetate and 11.5 ml of glacial acetic acid to 28.5 ml dist. water, resulting in a concentration of 3 M K^+ and 5 M CH_3COO^- .

standing the tube in an inverted position for 15 min. Any remaining moisture adhering to the centrifuge tube wall was removed with absorbant paper. The pellet dissolved readily in 1.5 ml TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 2.8 g CsCl (Boehringer, F.R.G) was added and dissolved at room temperature. A Beckman Bell-top Quick-Seal ultracentrifuge tube (13x32 mm) was loaded with 150 μ l ethidium bromide and the DNA sample was added to it. The sample was spun at 433,000xg for 16 h at 20°C in a Beckman TL 100 ultracentrifuge. The plasmid band was localised under UV illumination and recovered from the gradient by piercing a hypodermic needle through the wall of the centrifuge tube as described by Sambrook *et al.* (1989). The viscous plasmid DNA fraction was drained into an Eppendorf tube and ethidium bromide was removed by repeated (*ca.* six times) extraction with water-saturated butanol. The DNA sample was diluted with 3 volumes of double dist. water and DNA was precipitated with 0.6 volume of *iso*-propanol at 4°C¹¹ for 15 min. The precipitated DNA was pelleted in an Eppendorf centrifuge and dissolved in 40 μ l double dist. water. The DNA concentration was estimated spectrophotometrically at 260 nm (OD₂₆₀ of 1.0 \equiv 50 μ g·ml⁻¹ DNA).

2.20.2 cDNA isolation

To 40 μ l plasmid DNA solution 5 μ l 10x E₄ buffer (*Eco*RI incubation buffer, Amersham, U.K.) and 60 units (5 μ l) *Eco*RI (Amersham, U.K.) were added and incubated at 37°C for 3-5 h. The incubation mixture was then subjected to non-denaturing agarose gel electrophoresis as described in section 2.18.1. When the tracker dye had run half way electrophoresis was stopped and the gel was inspected under UV illumination. Small incisions were made just above and below the band corresponding to the cDNA insert (1.7 kb) excised from the plasmid. Suitable pieces of DEAE membrane (Schleicher & Schuell, F.R.G.), wetted in 1x TBE, were inserted

¹¹ CsCl precipitated if the DNA solution was stored at -20°C.

into the cuts in the gel by carefully widening the incisions with forceps. Electrophoresis was resumed for 15 min until the cDNA band moved onto the DEAE membrane. The membrane with the ethidium bromide stained cDNA was removed from the gel and transferred to an Eppendorf tube. The membrane was incubated with 400 μ l high salt buffer (10 mM Tris-HCl pH 8.0, 1.5 M NaCl, 1 mM EDTA) at 65°C until no fluorescence was detectable on the membrane (*ca.* 15 min). The high salt solution was diluted with 400 μ l TE and extracted once with phenol and once with chloroform. The phenol phase was re-extracted with 200 μ l TE. DNA was precipitated from the pooled aqueous phases with 0.6 volume *iso*-propanol at -70°C and dissolved in double dist. water to a concentration of 50 ng· μ l⁻¹.

2.20.3 Random prime labelling reaction

Extracted cDNA was denatured by heating at 90-100°C for 10 min and cooled on ice. The labelling reaction was carried out at room temperature. The following reagents were pipetted into an Eppendorf tube in the stated order.

33	μ l	double dist. water	
10	μ l	OLB (Table 2.20.2)	
2	μ l	BSA (10 mg·ml ⁻¹ stock)	(DNA grade, Sigma)
2	μ l	cDNA (\approx 100 ng)	
2	μ l	α -[³² P]CTP (\approx 20 μ Ci)	
1	μ l	Klenow-fragment (\approx 4 units) (Amersham)	
		(DNA polymerase I, large fragment)	
<hr/>			
50	μ l	random prime reaction mix	

The reaction time was 5 h and the reaction was stopped by adding 50 μ l TE. 1 μ l of the reaction mix was dried onto a Whatman GF/C glass fibre filter disc.

Unincorporated label was separated from the cDNA probe by the spun column procedure (Sambrook *et al.*, 1989). The bottom of a 1 ml disposable syringe was plugged with a small amount of glass wool. In the syringe a column (0.9 ml bed

volume) was prepared of Sephadex G-50 (Pharmacia) equilibrated with TE. An Eppendorf tube was decapped and inserted into a glass centrifuge tube (15 ml). The syringe was inserted into the centrifuge tube so that the tip was in the decapped Eppendorf tube. The assembly was spun at 1600xg for 4 min in a bench top centrifuge. More Sephadex G-50 was added until the packed volume was 0.9 ml. The random prime reaction mixture (100 µl) was applied to the column and centrifuged at exactly the same speed and for exactly the same time as before, collecting the 100 µl eluent from the syringe in the decapped Eppendorf tube. This step was repeated with 100 µl TE and the flow through was collected in the same Eppendorf tube. An aliquot (1 µl) labelled cDNA was dried onto a Whatman GF/C glass fibre filter disc. Both filter discs were counted using liquid scintillation (section 2.7.2) to determine the rate of incorporation.

Table 2.20.2 Preparation of OLB (oligo-labelling-buffer)

Solution 0		dATP, dTTP, dGTP (separate solutions)			
1.25 M Tris-HCl pH 8.0		0.1 M dNTP			
0.125 M MgCl ₂		3 mM Tris-HCl pH 7.0			
stored at 4°C		0.2 mM EDTA			
		stored at -20°C			
solution A		solution B		solution C	
1000 µl solution 0		2 M HEPES		hexanucleotides	
18 µl β-mercapto ethanol		titrated		50 OD ₂₆₀ units were	
5 µl dATP		to pH 6.6 with		suspended in 550 µl	
5 µl dGTP		4 M NaOH		TE resulting in	
5 µl dTTP		stored at 4°C		90 OD ₂₆₀ units per ml	
stored at -20°C				stored at -20°C	
OLB					
solutions A:B:C in ratio					
10:25:15					
stored at -20°C					

2.21 Statistical analysis

All methods of statistical analysis, and the required distribution tables were taken from Rees (1985).

2.21.1 Mean, variance, standard deviation, and standard error

The arithmetic mean of a sample was calculated as the sum of the observations divided by the number of observations.

$$\bar{x} = (\Sigma x)/n = (x_1 + x_2 + \dots + x_n)/n$$

The geometric mean was determined when data were derived from ratios of measurements, e.g. specific enzyme activity (= quotient of enzyme activity and protein content).

$$\bar{x} = \sqrt[n]{x^n} = (x_1 \cdot x_2 \cdot \dots \cdot x_n)^{(1/n)}$$

The variance is defined as the quotient of the sum of the squares of all deviations from the mean and the number of observations reduced by one.

$$s^2 = (\Sigma (x - \bar{x})^2)/(n-1) = (\Sigma x^2 - (\Sigma x)^2/n)/(n-1)$$

The square root of the variance gives the standard deviation which is a measure of the distribution of the observations around the mean of the sample.

$$\sigma = \sqrt{s^2} = ((\Sigma (x - \bar{x})^2)/(n-1))^{(1/2)}$$

In normally distributed samples 68.27% of all observations are grouped between $\bar{x} - \sigma$ and $\bar{x} + \sigma$, 95.45% between $\bar{x} \pm 2 \sigma$, and 99.37% between $\bar{x} \pm 3 \sigma$.

The meaningfulness of the mean can be estimated by the standard error. It is defined as quotient of the standard deviation and the square root of the number of observations.

$$SE = \sigma/\sqrt{n}$$

The standard error is a measure of how close the mean of the sample is to the true mean of the population. The analysis of the standard error is similar to the analysis of the standard deviation from which it derived, i.e. the means of two samples taken from the same population should not differ by more than the standard error in 68.27% of the observations.

2.21.2 Comparison of the means of two samples

Observed differences between means were assessed using "Student's t-test", as it is suitable for the small sample sizes used in this investigation.

The first sample contained n_1 observations and had a mean \bar{x}_1 , and a variance s^2_1 . In the second sample the corresponding values were n_2 , \bar{x}_2 , and s^2_2 . The null hypothesis was tested that the true means μ_1 and μ_2 were equal. The alternative hypothesis was that there was a difference, in one direction or the other. The chosen significance level was 5%.

- 1) $H_0 : \mu_1 = \mu_2$

- 2) $H_1 : \mu_1 \neq \mu_2$

- 3) 5% significance level

- 4) Calc $t = (\bar{x}_1 - \bar{x}_2) / \sqrt{s^2 \cdot (1/n_1 + 1/n_2)}$

The pooled estimate of variance (s^2) was calculated from the sample variances:

$$s^2 = ((n_1 - 1) \cdot s^2_1 + (n_2 - 1) \cdot s^2_2) / (n_1 + n_2 - 2)$$

5) The table of "Student's" t-distribution was then referred to at (n_1+n_2-2) degrees of freedom. If $|t_{\text{Calc}}| < t_{\text{Tab}}$, then H_0 was not rejected. The sample means were not significantly different (5% level)

2.21.3 Regression analysis

Regression analysis was performed to determine if there was an association between two measurements, x and y . The assumption was made that the true means of the dependant variable y for each value of the independant variable x lay on a straight line. This line shows the linear regression of y on x , and is termed the regression line.

$$y = \alpha + \beta \cdot x$$

where β is the regression coefficient of y on x . It is zero if y and x are not related.

The true regression coefficient (β) was estimated by b :

$$b = (\Sigma(x-\bar{x}) \cdot (y-\bar{y})) / (\Sigma(x-\bar{x})^2)$$

and the true constant α was estimated by a :

$$a = \bar{y} - b \cdot \bar{x}$$

The fitted regression line is thus:

$$y = a + b \cdot x$$

3. RESULTS

3.1 Rationale

Aspects of the regulation of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) in *Capsicum frutescens* cell cultures have been studied in this thesis.

It is well known that PAL plays a key role in phenylpropanoid metabolism in a variety of plant species (Hanson and Havir, 1981; Hahlbrock and Scheel, 1989) and it is considered to be an important link between primary and secondary metabolism in plants (Creasy, 1987).

However, PAL is not considered to be a key regulatory enzyme in the biosynthesis of capsaicin (Lindsey, 1986), but increased incorporation of radiolabelled precursors have been observed during elicitation of *Capsicum* cell cultures and this was preceded by elevated levels of PAL activity (Holden *et al.*, 1988a).

Accordingly, the regulation of PAL in *Capsicum* cell cultures during treatment with a fungal elicitor was studied and the developmental stage of the cell cultures at which PAL responded strongest to elicitation was determined (section 3.3). This was preceded by a detailed characterisation of the growth cycle of *Capsicum frutescens* cell cultures (section 3.2).

Attention was then focussed on time course experiments investigating changes in PAL activity following elicitation of *Capsicum* cultures (section 3.4).

Subsequently, PAL protein sub-units were identified and the regulation of PAL activity at a translational level was studied (section 3.5). Levels of PAL mRNA, detected by Northern Blotting, were correlated with changes in PAL activity and PAL protein (section 3.6).

Finally, the synthesis and accumulation of soluble phenolic compounds and PAL levels were followed during elicitation of *Capsicum* suspension cultures and a comparison was made with non-elicited cultures.

3.2 Characterisation of the growth cycle of *Capsicum frutescens* cell cultures

The following experiments were designed to characterise the growth cycle of *Capsicum frutescens* cell cultures.

Cell suspensions were initiated and maintained in S+H medium (Table 2.3.1) as described in section 2.3.1. The method of choice for describing culture growth was the determination of packed cell volume (PCV). The PCV was expressed as a percentage of the total culture volume. Both volumes were determined simultaneously by spinning 50 ml suspension cultures in graduated centrifuge tubes at 750xg for 5 min using a table top centrifuge.

3.2.1 Slow growing *Capsicum* cultures

The *Capsicum* cell suspensions tended to form compact aggregates and exhibited slow growth. The growth curve for this type of cultures is shown in Fig. 3.2.1.

Attempts to break up larger cell aggregates with a sterile spoon spatula before sub-culture proved unsuccessful. The lag phase of these slow growing cultures lasted *ca.* 21 d and the stationary phase had not been reached after 34 d. As a comparison, the *Capsicum* suspensions described by Holland (1989) reached the stationary phase after 21 d.

3.2.2 Selection of fast growing suspensions

Gradually, cultures were selected for fast growth. Slow growing cultures with large, compact cell aggregates were discarded and only suspensions with smaller aggregates were sub-cultured. Also, some new suspensions were initiated by inoculation of S+H medium (Table 2.3.1) with fresh friable callus.

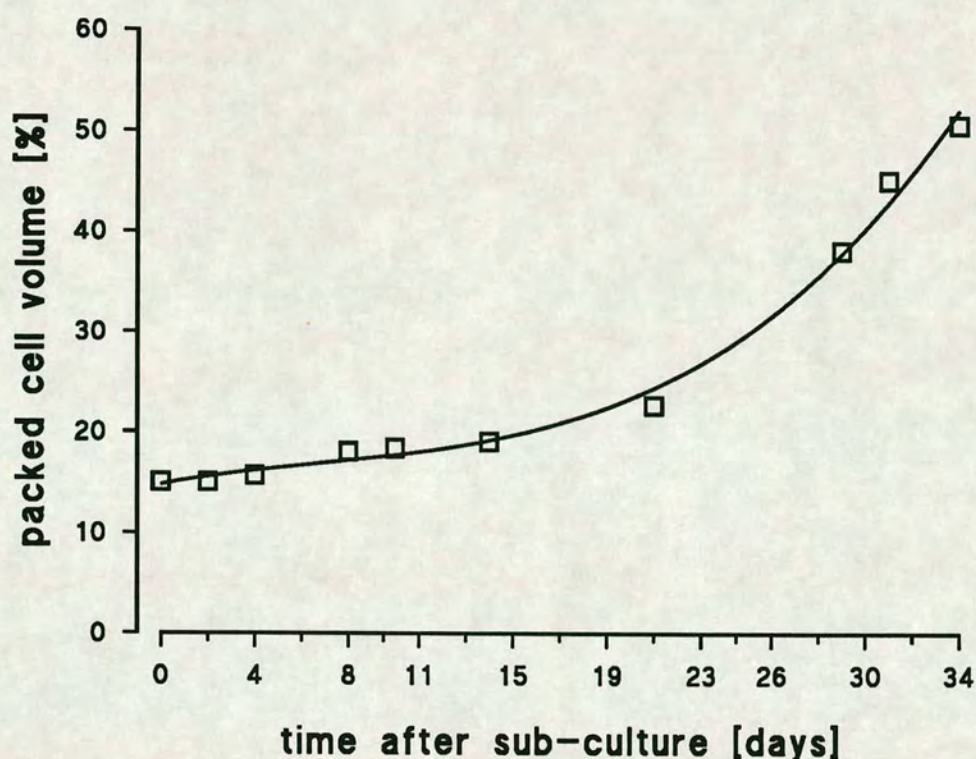


Figure 3.2.1 Growth curve of *Capsicum frutescens* cell cultures before selection for fast growing, fine suspensions. Plotted data represent arithmetic means of six replicates.

Larger aggregates were removed from the suspensions by filtering through nylon mesh (pore size 600 μm , section 2.3.1) over six subsequent sub-cultures. The selection procedure resulted in suspension cultures with small cell aggregates and a shorter culture cycle. The growth curve of these cultures is shown in Fig. 3.2.2.

Arithmetic means of the PCV raw data were plotted against time and a curve was fitted by non-linear regression analysis. The large standard residue of the PCV value at time 12 d is due to one replicate of the raw data.

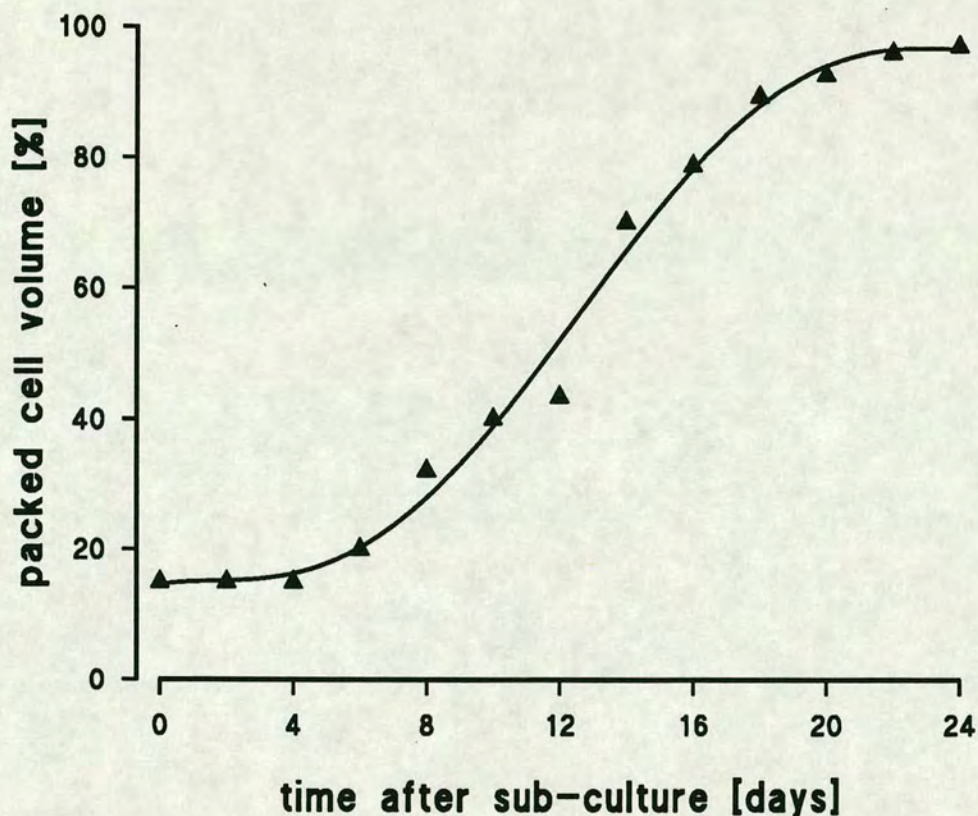


Figure 3.2.2 Growth curve of *Capsicum frutescens* cultures selected for fast growth and the formation of fine suspensions. Plotted data represent arithmetic means of three replicates each.

3.2.3 Growth patterns in *Capsicum* cell cultures

3.2.3.1 Lag phase

The lag phase is the initial period of a batch culture when no increase in biomass is apparent (King and Street, 1973). PCV was used in this investigation as an indicator for biomass.

During the time period $t_0 - t_4$ no increase in PCV was detected (Fig. 3.2.2) and the relative growth rate $[(\log_e(\text{PCV}_{t_2}) - \log_e(\text{PCV}_{t_1})) / (t_2 - t_1)]$ was consequently zero. It can be concluded, that the growth cycle of *Capsicum* cell suspensions has a lag phase during the first four days after subculture.

3.2.3.2 Exponential phase

Cell cultures exhibit a period of exponential growth following the lag phase of the growth cycle.

Mathematically, culture growth during this period can be described by an exponential function. The increase in biomass (PCV) within a certain time, t , is expressed as follows:

$$PCV_t = e^{\mu \cdot t} \cdot PCV_0 \quad (1)$$

where PCV_0 = PCV initially and μ = specific growth rate. The specific growth rate is constant during the exponential growth phase

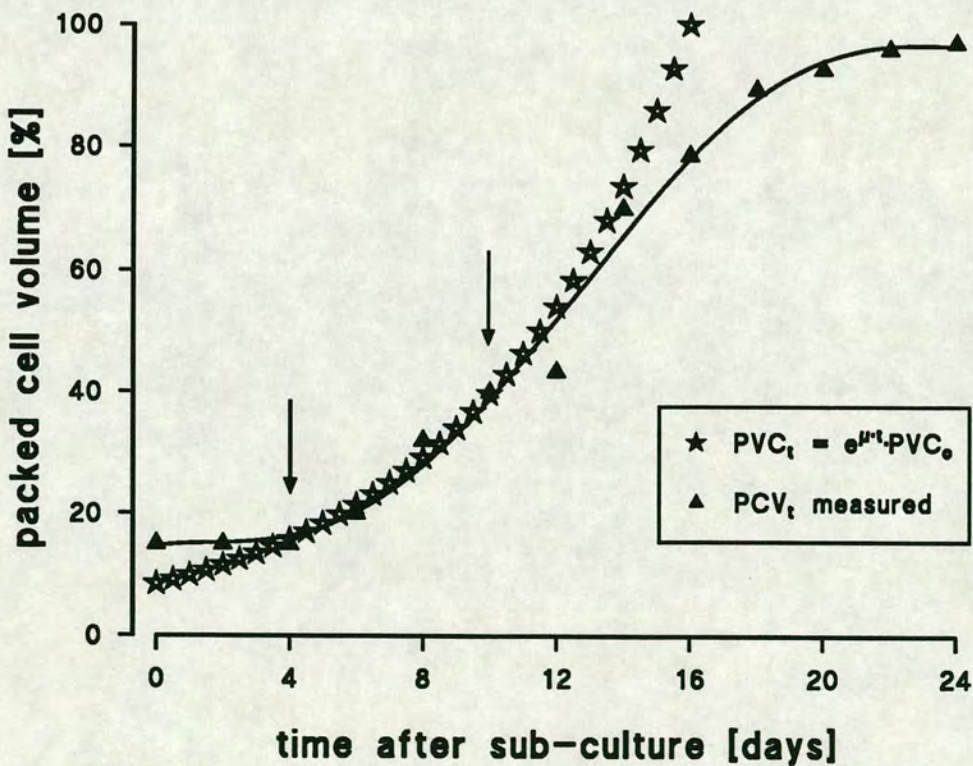


Figure 3.2.3 *Capsicum frutescens* growth curve and calculated exponential growth curve. The part of the *Capsicum* growth curve that follows exponential growth is indicated by arrows.

In Fig. 3.2.3 calculated data points for an exponential growth curve (equation (1)) were plotted against time. The growth curve determined by PCV measurements and the calculated exponential growth curve are congruent between day 4 and day 10 of the growth cycle.

Logarithmic transformation of equation (1) results in an equation describing a straight line:

$$\log_e(\text{PCV}_t) = \mu \cdot t + \log_e(\text{PCV}_0) \quad (2)$$

Therefore, plotting values of t (abscissa) and of $\log_e(\text{PCV})$ (ordinate), a straight line is obtained for the exponential growth phase (when $\mu = \text{constant}$), with slope = μ (specific growth rate)

In Fig. 3.2.4 $\log_{10}(\text{PCV})$ is plotted against time. The linear regression between $t=4$ d and $t=10$ d allows the calculation of the specific growth rate by rearranging equation (2):

$$(\log_e(\text{PCV}_t) - \log_e(\text{PCV}_0)) / t = \mu \quad (3)$$

The slope of the linear regression in Fig. 3.2.4 gives $\mu/2.303$ because $\log_{10}(\text{PCV})$ was plotted instead of $\log_e(\text{PCV})$. The specific growth rate was determined to be $0.155 [\text{d}^{-1}]$.

PCV_0 in equation (3) is not identical with the PCV value measured at t_0 . It represents the initial PCV of the calculated exponential growth curve (equation (1)). The calculated curve is congruent with the experimentally established growth curve only between day 4 and day 10. Therefore, PCV_0 calculated is not identical with PCV_0 measured.

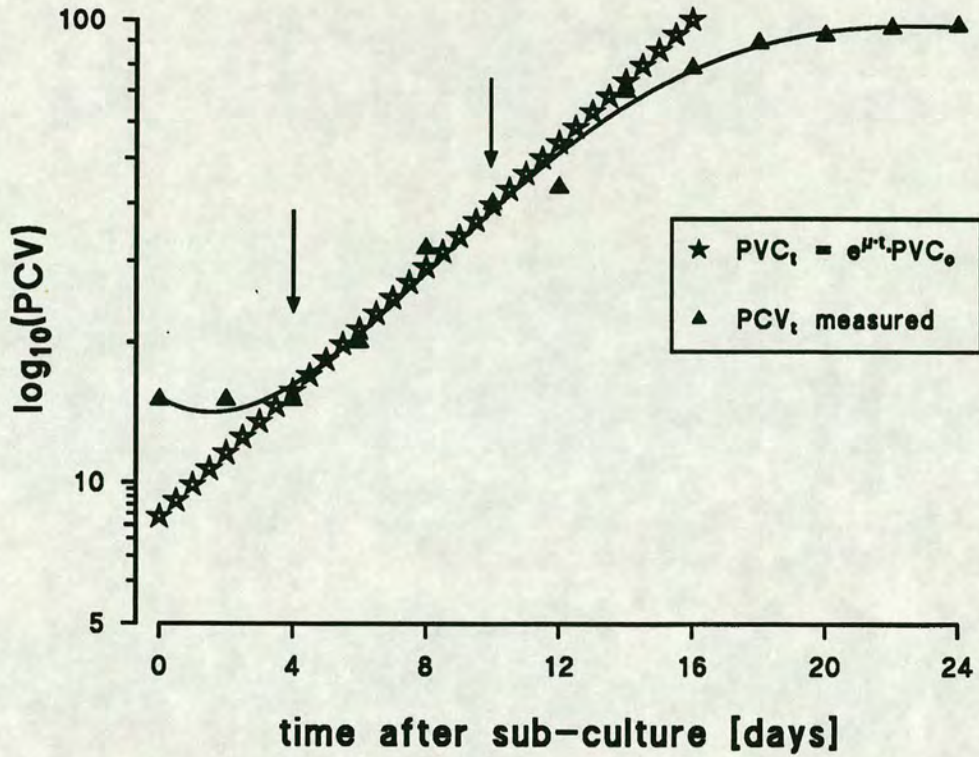


Figure 3.2.4 Semi-logarithmic plot of *Capsicum* growth curve and calculated exponential growth curve. The exponential phase of the *Capsicum* growth curve is indicated by arrows.

The specific growth rate obtained from the linear regression in Fig. 3.2.4 is identical with the relative growth rate for this part of the growth curve. The relative growth rate is calculated by exchanging PCV_0 (extrapolated) with PCV_{t1} (measured). Equation (3) changes accordingly to:

$$(\log_e(PCV_{t2}) - \log_e(PCV_{t1})) / (t_2 - t_1) \quad (4)$$

Between day 4 and day 10 the relative growth rate (equation (4)) of the fitted curve of the *Capsicum* growth cycle is constant and identical with the specific growth rate (equation (3)) of the calculated exponential growth curve. During that period of time (4 d to 10 d after sub-culture) the growth curve of *Capsicum* cell cultures has an exponential phase.

3.2.3.3 Linear phase

The exponential phase of growth is succeeded by a linear phase during which the increase in PCV per unit time is constant.

In Fig. 3.2.5 the PCV increments per 2 days of culture are plotted as histograms underneath the *Capsicum* growth curve.

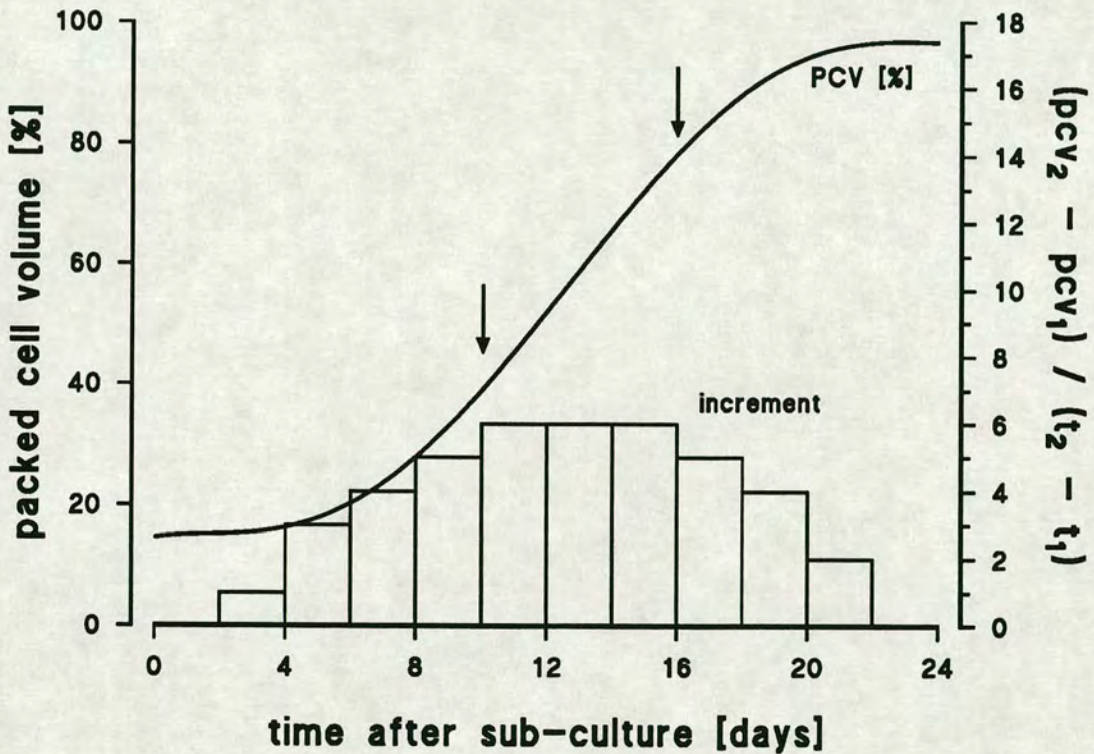


Figure 3.2.5 *Capsicum frutescens* growth curve and PCV increments per 2 days of culture. Arrows indicate the linear growth phase.

Between day 10 and day 16 the PCV increase $((PCV_{t_2} - PCV_{t_1}) / (t_2 - t_1))$ is constant and the growth curve has a linear phase. The relative growth rate is positive but decreases from its maximal value (exponential phase) during the linear phase (data not shown).

3.2.3.4 Progressive deceleration phase

Following the linear growth phase, the PCV increments (Fig. 3.2.5) decrease until no net synthesis of biomass is detectable. During this phase of the growth cycle the relative growth rate decelerates more progressively than during the linear phase until it reaches zero at day 22. The progressive deceleration of the relative growth rate results in the observed decrease in PCV increments. The *Capsicum* growth cycle has a progressive deceleration phase between day 16 and day 22 after sub-culture.

3.2.3.5 Stationary phase

The stationary phase is the terminal phase of a batch culture growth cycle where no net increase in biomass is apparent. PCV measurements at day 22 and day 24 of the *Capsicum* growth cycle are not significantly different as estimated by "Student's t" analysis (statistical analysis was performed using the original data (in ml)). Both PCV values however, are significantly different (1% level) from the measurements at day 20. It can be concluded, that *Capsicum* cell suspensions reach the stationary phase between day 20 and day 22 after subculture.

3.2.3.6 Characterisation of the *Capsicum* growth cycle

The growth cycle of *Capsicum frutescens* suspension cultures was characterised in section 3.2.3 and the following growth phases were identified (Fig. 3.2.6).

- (1) Lag phase (0 d - 4 d)
- (2) Exponential phase (4 d - 10 d)
- (3) Linear phase (10 d - 16 d)
- (4) Progressive deceleration phase (16 d - 21 d)
- (5) Stationary phase (21 d onwards)

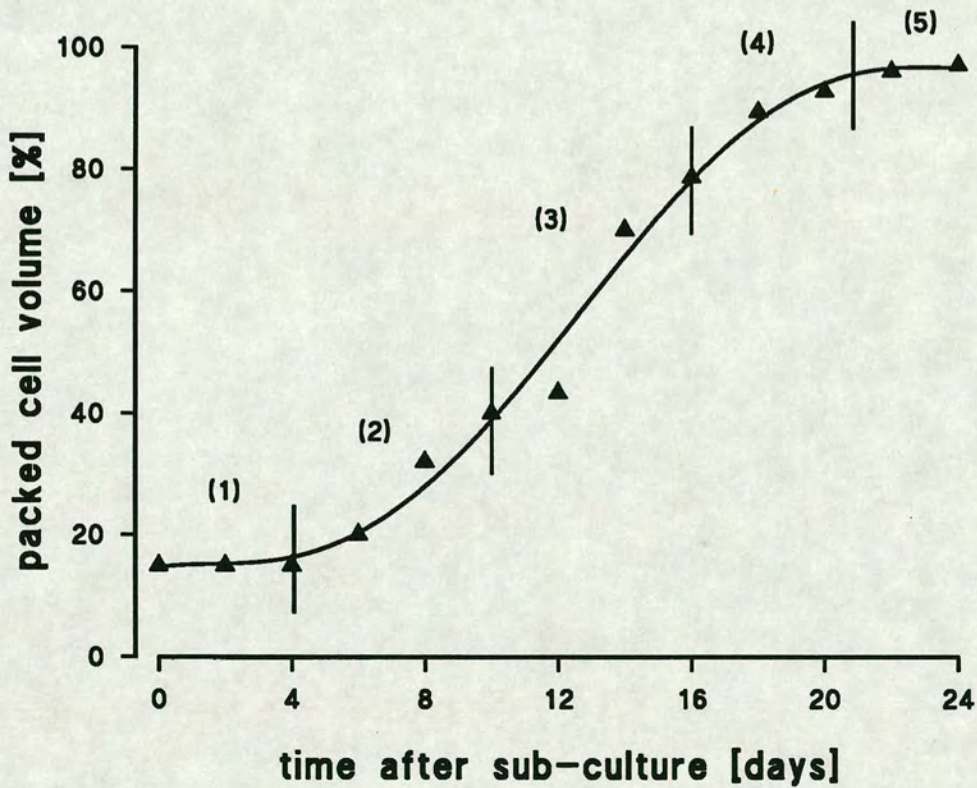


Figure 3.2.6 Growth phases of the *Capsicum frutescens* cell suspension growth cycle. (1) Lag phase, (2) Exponential (logarithmic) phase, (3) Linear phase, (4) Progressive deceleration phase, (5) Stationary phase

3.2.4 Changes in culture medium pH during the growth cycle

The pH of fresh culture medium, originally adjusted to pH 5.7, dropped to pH 5.6 after autoclaving. The pH of the culture medium dropped further after inoculation with cells and their adhering spent medium (Fig. 3.2.7).

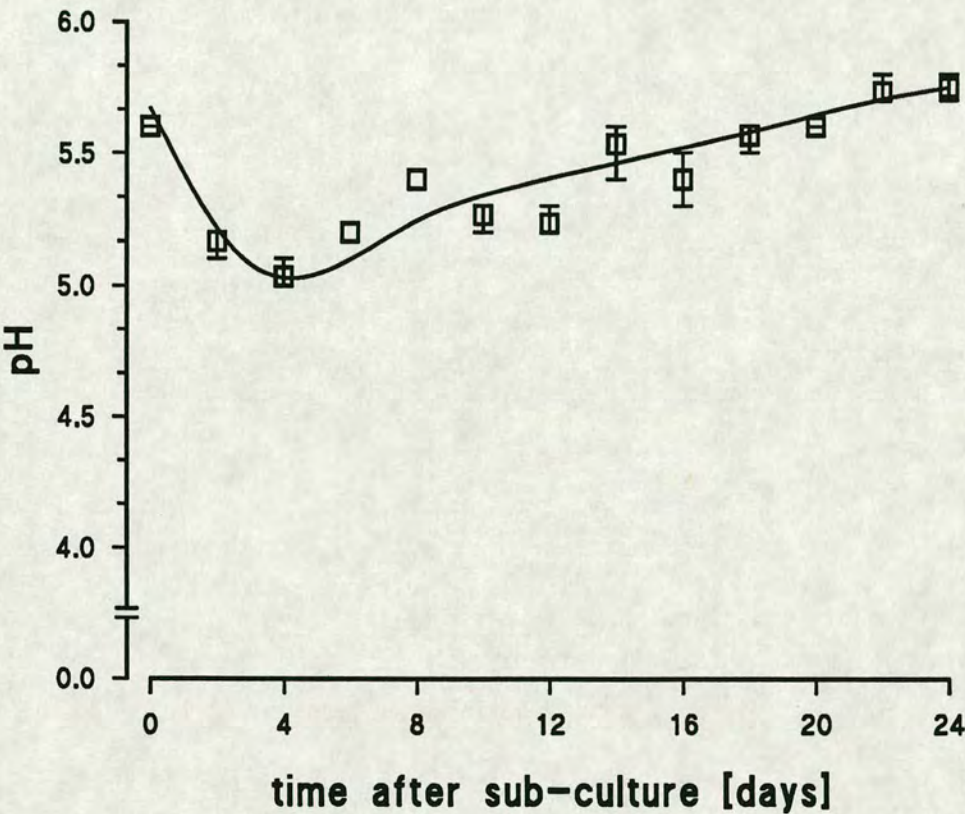


Figure 3.2.7 The pH of the culture medium of fast growing *Capsicum frutescens* cell suspensions. Each data point represents the arithmetic mean of three replicates \pm SE

The pH reached its lowest value (pH 5.0) *ca.* 4 d after sub-culture. Subsequently, the pH of the culture medium increased slowly over the rest of the culture cycle. The minimum pH value coincided with the end of the lag phase of growth as indicated by arrows in Fig. 3.2.8. The pH of the spent medium at the end of the growth cycle

(pH 5.8) was only slightly higher than the pH of fresh, pre-autoclaved medium (pH 5.7).

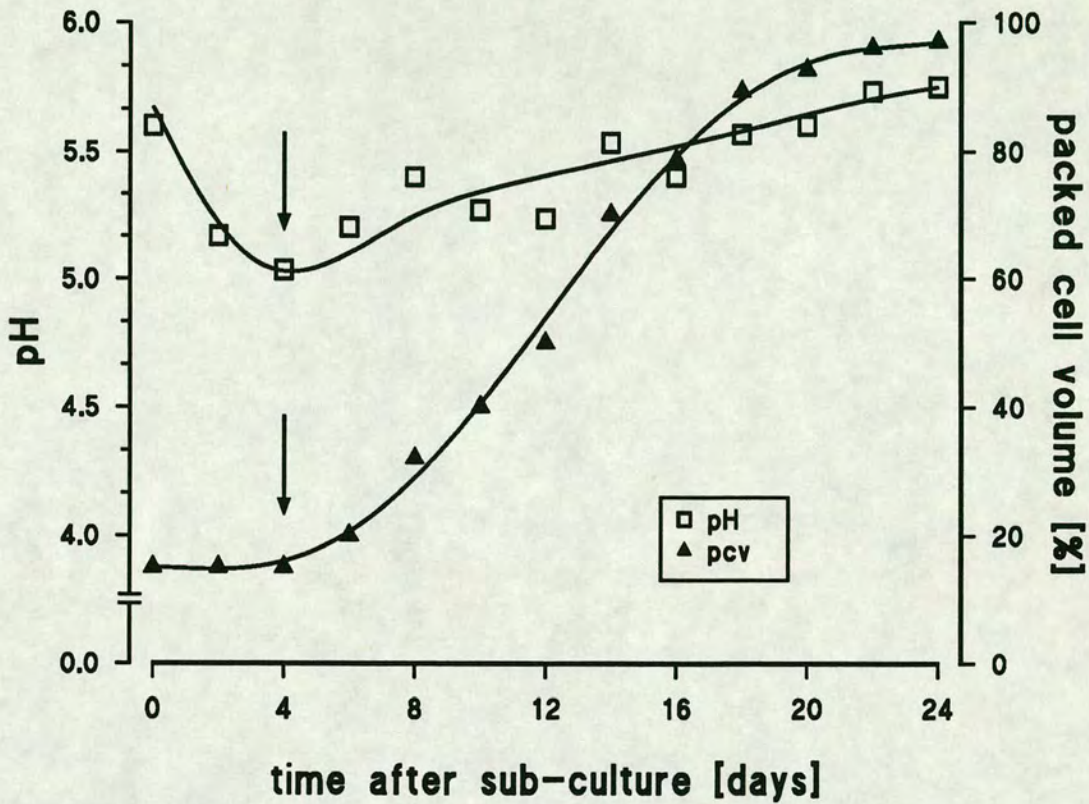


Figure 3.2.8 Growth curve of *Capsicum* suspension cultures and pH of culture medium. Each data point represents the arithmetic mean of three replicates.

The reasons for the observed changes in pH during the growth cycle are complex and cannot be fully explained without further investigation. However, it is likely that changes in pH reflect a combination of nutrient uptake, release of secondary metabolites, and release of substances due to cell death.

3.2.5 Comparison of PCV and fresh weight as indicators for culture growth

Packed cell volume was chosen as the method for following cell culture growth because it is easy to determine. Also, PCV determination is non-destructive when performed under aseptic conditions. The accuracy of PCV as an indicator of culture growth can be gauged when compared with the determination of fresh weight. Both methods are of similar ease of performance. As shown in Fig. 3.2.9 the fitted curves for fresh weight and PCV run approximately parallel for the entire growth cycle. Therefore, it can be concluded that both methods are equally suitable for following the growth of *Capsicum frutescens* cell cultures.

As routine, PCV was the preferred method for the reasons stated above and because of the smaller variation among replicates. The average standard error of the arithmetic mean of the fresh weight data was *ca.* 5% of the value of the mean compared with *ca.* 2% for cell volume (in ml) determinations (data not shown). The larger differences observed between fresh weight replicates could be due to different amounts of liquid adhering to the cell surfaces after vacuum filtration. Although, suction was applied for the same period of time to all samples differences in the amount of liquid retained by the cultures presumably exist and were not taken into account.

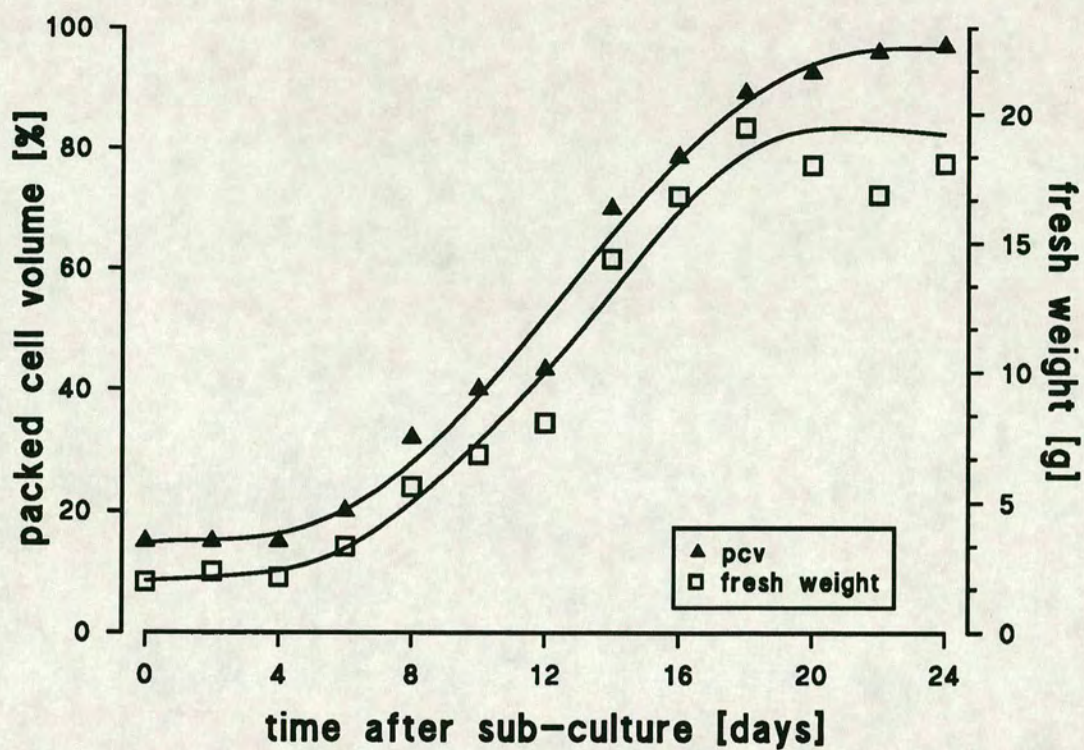


Figure 3.2.9 The determination of fresh weight and packed cell volume as indicators of culture growth. Each data point represents the arithmetic mean of three replicates.

3.2.6 Summary

The experiments described in section 3.2 were conducted to characterise the growth cycle of *Capsicum frutescens* cell cultures. The determination of packed cell volume (PCV) was used to establish the growth curve of *Capsicum* suspensions. PCV determination was found to be superior to fresh weight determination, a method of similar speed and ease of performance.

Fast growing suspensions with small cell aggregates were established by consequent selection.

Changes in pH during the growth cycle were determined and it was shown that the lowest pH value coincided with the start of the exponential growth phase of the suspensions *ca.* 4 d after sub-culture. It was anticipated that pH could effect the uptake of precursors by cells as demonstrated by Holland (1989).

The established growth curves now form a firm basis for subsequent studies on the effects of a fungal elicitor on PAL activity.

3.3 Changes in PAL activity during elicitation at various developmental stages of *Capsicum* cell suspensions

Elicitation of *Capsicum frutescens* suspension cultures with aqueous preparations of *Gliocladium deliquescens* spores is known to lead to a rapid and transient increase in PAL activity (Holden *et al.*, 1988b).

In section 3.2 the growth cycle of the *Capsicum* cultures established during this investigation was characterised in detail. The experiments described in this section were designed to determine the developmental stage at which *Capsicum* cell suspensions respond most strongly to the fungal elicitor as evidenced by changes in PAL activity.

3.3.1 Elicitation of *Capsicum* cell cultures

Aqueous extracts of a fungal elicitor were prepared as described in section 2.4.1 and the total carbohydrate concentration was estimated (section 2.4.2). Regardless of the amount of cells at the various developmental stages of the cultures all experimental flasks were treated with the same amount of elicitor preparation.

Aqueous extracts of *Gliocladium deliquescens* spores containing *ca.* 2 mg carbohydrate (glucose equivalents, see section 2.4.2) in a volume of 1-1.5 ml were used to elicit 50 ml *Capsicum* cell suspensions. This particular elicitor concentration was chosen because it represents the amount of carbohydrate released from approximately 30 mg spores. Holden *et al.* (1988b) established that the treatment of 50 ml *Capsicum* suspensions with 30 mg *Gliocladium deliquescens* spores resulted in the highest transient induction of PAL activity. Cell suspensions at various developmental stages were elicited for 8 h and the controls were treated with an equivalent volume of water for the same length of time.

3.3.2 Specific PAL activity in response to elicitation of cell cultures

PAL activity was extracted and assayed as described in sections 2.5.1 and 2.5.2, respectively. Soluble protein concentration was estimated as outlined in section 2.6. Specific PAL activity was calculated as described in section 2.5.3.

The specific activities of elicited and control cultures were determined at various developmental stages. The results are listed in Table 3.3.1. The increase in specific activity due to elicitation is expressed as a quotient of the specific activities of elicited and control cultures.

Table 3.3.1 Cell cultures at various developmental stages, indicated by the packed cell volume (PCV), were elicited for 8 h using aqueous preparations of *Gliocladium deliquescens* spores. Controls were treated with an equivalent volume of water.

PCV [%]	specific PAL activity [$\mu\text{kat} \cdot (\text{kg protein})^{-1}$]		-fold increase
	control	elicited	
19.0	46.7 \pm 6.0	301.6 \pm 23.7	6.5
22.6	30.5 \pm 7.6	151.2 \pm 4.7	5.0
38.0	17.9 \pm 11.9	185.5 \pm 11.5	10.4
90.0	4.1 \pm 2.9	55.2 \pm 6.2	13.5

The experimental flasks were taken from more than one culture cycle, therefore the developmental stage was expressed as PCV rather than time after sub-culture.

The PCV data (19-90%) presented in Table 3.3.1 correspond approximately to the following developmental stages of the *Capsicum frutescens* cell culture growth cycle

(Fig. 3.2.1 and Fig. 3.2.2). Lag phase, exponential growth phase, start of linear growth phase, stationary growth phase.

The data obtained from un-elicited (control) cell cultures suggest that specific PAL activity stayed at a low level with a tendency to decrease during the culture cycle. These findings do not concur with the data obtained by Holland (1989) who found that the specific PAL activity increased towards the end of the growth cycle of *Capsicum* cell cultures. PAL activities reported for other plant cell culture systems stay at a low basic level, except for a transient increase after dilution into fresh medium during sub-culture (Hahlbrock and Wellmann, 1970; Hahlbrock and Schröder, 1975; Bevan and Northcote, 1979; Ozeki and Komamine, 1985). A second peak in PAL activity was observed during the growth cycle when the cells were cultured in an inductive medium (Bevan and Northcote, 1979; Ozeki and Komamine, 1985). This second peak occurred at the late linear phase and correlated with the start of increased phenolic production and the exhaustion of nitrogen in the medium. However, PAL activity returned to a base level at early stationary phase (Cvikrová *et al.*, 1988; Westcott and Henshaw, 1976).

The objective of this investigation was to study the response of PAL activity to elicitation at different developmental stages.

All cultures responded to 8 h elicitation with an increase in specific PAL activity (Table 3.3.1). The *Capsicum* cultures with the highest basal PAL activity showed the highest PAL activity when elicited. The lowest PAL activity in the controls resulted in the lowest activity upon elicitation.

This feature is emphasised by the fitted curves through the data points in Fig. 3.3.1. These curves do not represent regressions but merely display tendencies.

An exception to the established correlation was the data pair at 22.6% PCV. The specific PAL activity determined for the controls at this developmental stage lies within the range of the tendency line fitted in Fig. 3.3.1. The PAL activity of elicited

cultures at this part of the growth curve however, did not match with any other data obtained in respect to absolute amount of PAL activity and the ratio of activities of elicited and un-elicited cultures. It was therefore considered an artefact and disregarded in this analysis.

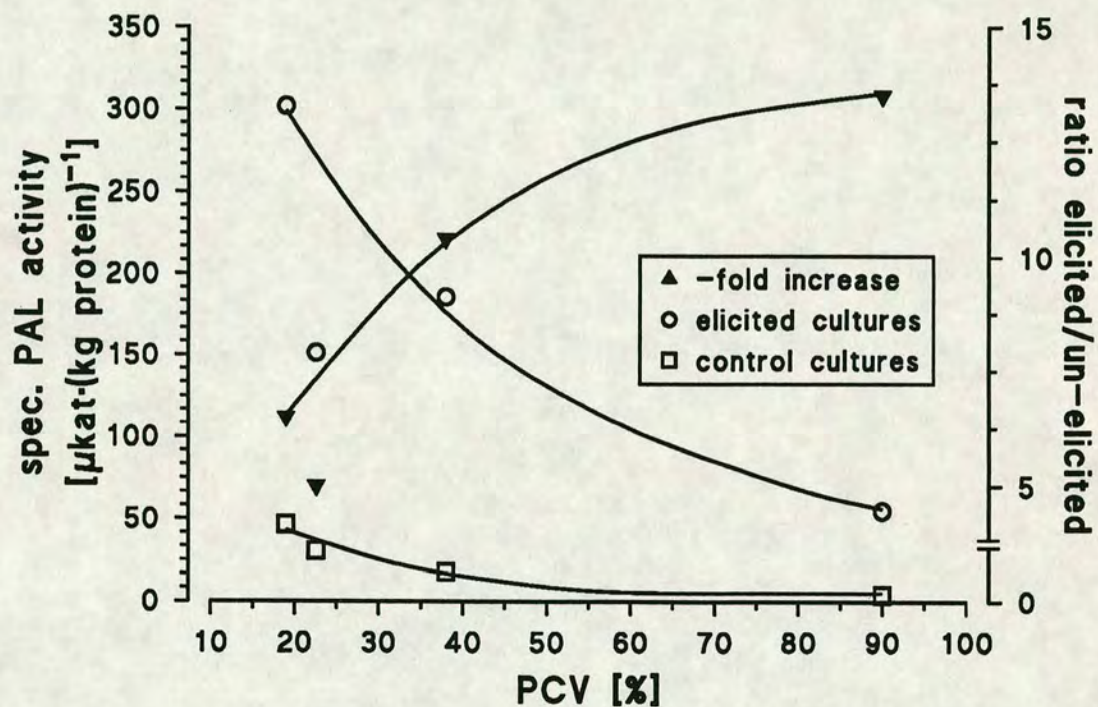


Figure 3.3.1 Specific PAL activities of elicited cultures and controls, and increases in specific PAL activities as response to elicitation. The fitted curves indicate trends, not regressions. Three replicates per data point

The absolute amounts of detectable PAL activity measured in elicited and un-elicited cultures decreased during the growth cycle (Fig. 3.3.1). The activities determined during the lag phase of culture growth were highest, and the activities determined at the stationary phase were lowest. However, the response of the cultures to elicitation showed an inverse correlation. Cultures with the lowest basal levels of PAL activity responded with the strongest increase in specific PAL activity during

elicitation (13.5 times control level). Although, the cultures during the lag phase of the growth cycle responded to elicitation with an increase in specific PAL activity up to $301.6 \mu\text{kat} \cdot (\text{kg protein})^{-1}$, the highest activity measured in this investigation, this increase represents only 6.5 times the activity of the control level at this developmental stage.

3.3.3 Consequences for further elicitation experiments

The trend of increase in specific PAL activity as a response to elicitation is indicated as a tendency line in Fig. 3.3.1. The response seemed to be strongest when the cultures reached the stationary phase of the culture cycle.

The down-turn of growth at the stationary phase often corresponds with the onset of synthesis of secondary metabolites (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1985a). The *Capsicum* cultures used in this investigation reached the stationary phase *ca.* 21 d after sub-culture (section 3.2.3.5). At this developmental stage the pH of the culture medium is approximately the same as the pH of fresh medium (section 3.2.4).

The stationary phase of the culture cycle of *Capsicum* cell suspensions was chosen as the developmental stage where all further elicitation experiments would be carried out for the following reasons. (1) The specific PAL activity showed the strongest response to elicitation. (2) The down-turn of growth creates favourable conditions for secondary metabolism which was to be investigated in conjunction with fungal elicitation. (3) The predictability of the pH of the culture medium was of importance for precursor feeding experiments.

The earliest date the *Capsicum* cell suspensions reached the stationary phase of growth was 21 d after sub-culture. Therefore, for all further elicitation experiments 21 d old cultures were used.

3.3.4 Summary

The experiments described in this section were designed to determine the developmental stage of the *Capsicum frutescens* cell cultures when fungal elicitation triggered the highest response in specific PAL activity.

The experimental results indicate that the absolute amounts of PAL activity in elicited and un-elicited (control) cultures decreased during the culture cycle from lag phase to stationary phase. The response of specific PAL activity to elicitation, expressed as quotient of PAL activities of elicited and un-elicited cultures, increased at the same time. Although, the amounts of specific PAL activities at the stationary phase were the lowest, elicitation induced the strongest response in PAL activity (13.5 times the control level). For this reason, and because of possible favourable conditions for secondary metabolism and the predictability of the medium pH, the stationary phase of the culture cycle was chosen as the developmental stage of *Capsicum* cultures when all further elicitation experiments were carried out.

The characterisation of the *Capsicum frutescens* culture cycle (section 3.2) and the investigation of the induction of PAL activity by elicitation during the culture cycle (this section) provided the necessary background information for the elicitation time course experiments described in section 3.4.

3.4 Induction of PAL activity during elicitation of *Capsicum frutescens* cell cultures

Detailed studies of changes in PAL activity, following elicitation of *Capsicum* cultures at various stages of the culture cycle (section 3.3), revealed that the cell cultures responded strongest to elicitation, indicated by increased PAL activity, towards the end of the culture cycle when PAL activity of untreated cells was lowest. Therefore, the stationary phase of culture growth was chosen as the developmental stage to study in detail fungal elicitation of *Capsicum* cultures. The earliest possible time for the *Capsicum* cell cultures used in this investigation to reach the stationary phase was 21 d after sub-culture (section 3.2.3.5).

For the preliminary studies on PAL activity induction in section 3.4.1 however, *Capsicum* cell cultures of an earlier developmental stage (PCV 22.6%) were used.

3.4.1 Comparison of PAL activities in elicited and un-elicited *Capsicum* cultures

Capsicum cell cultures (PCV 22.6%) were elicited using aqueous extracts of *Gliocladium deliquescens* spores as described in section 3.3.1. Control cultures were treated with an equivalent volume of water. Cells were harvested by vacuum filtration at various time intervals after the addition of the fungal elicitor. PAL activity was extracted immediately from harvested cells as described in section 2.5.1 and specific activity of the enzyme was determined (section 2.5.3).

PAL activity estimated from cell suspensions treated for 2 h with fungal elicitor was not significantly different from that of untreated cell cultures (Table 3.4.1 and Fig. 3.4.1)

Between 2 h and 8 h after addition of the fungal elicitor PAL activity in elicited cultures increased sharply (Fig. 3.4.1). PAL activity of un-elicited (control) cultures did not change. After 16 h elicitation PAL activity levels were still elevated compared

to control cultures. Specific PAL activity 16 h after addition of the elicitor was *ca.* 25% lower than the maximum activity measured after 8 h elicitation.

Table 3.4.1 Comparison of specific PAL activities extracted from elicited and control *Capsicum* cultures. Enzyme activity was determined using crude enzyme extracts and 40-55% (NH₄)₂SO₄ fractions. Elicitation times were 2 h, 8 h, and 16 h.

time	specific PAL activity [μ kat·(kg protein) ⁻¹] \pm SE			
	crude enzyme extract		(NH ₄) ₂ SO ₄ fraction	
	control	elicited	control	elicited
2 h	27.4 \pm 1.4	23.2 \pm 1.3	29.6	27.3
8 h	30.5 \pm 7.6	151.2 \pm 4.7	32.6	233.8
16 h	23.0 \pm 2.2	113.6 \pm 10.8	25.2	184.0

The results presented in this section demonstrate that treatment of *Capsicum frutescens* cell suspensions with aqueous preparations of *Gliocladium deliquescens* spores leads to an increase in PAL activity. Control cultures were treated as elicited cultures except that the aqueous extract of fungal elicitor was replaced by an equivalent volume of water. The volume (1-1.5 ml) added to elicited and un-elicited cultures was too small to have an effect on PAL activity in the controls. No statistically significant change in specific PAL activity was observed in un-elicited cultures throughout the entire period of elicitation. It can be concluded, that the observed increase in PAL activity of elicited cultures was caused by the presence of the fungal elicitor.

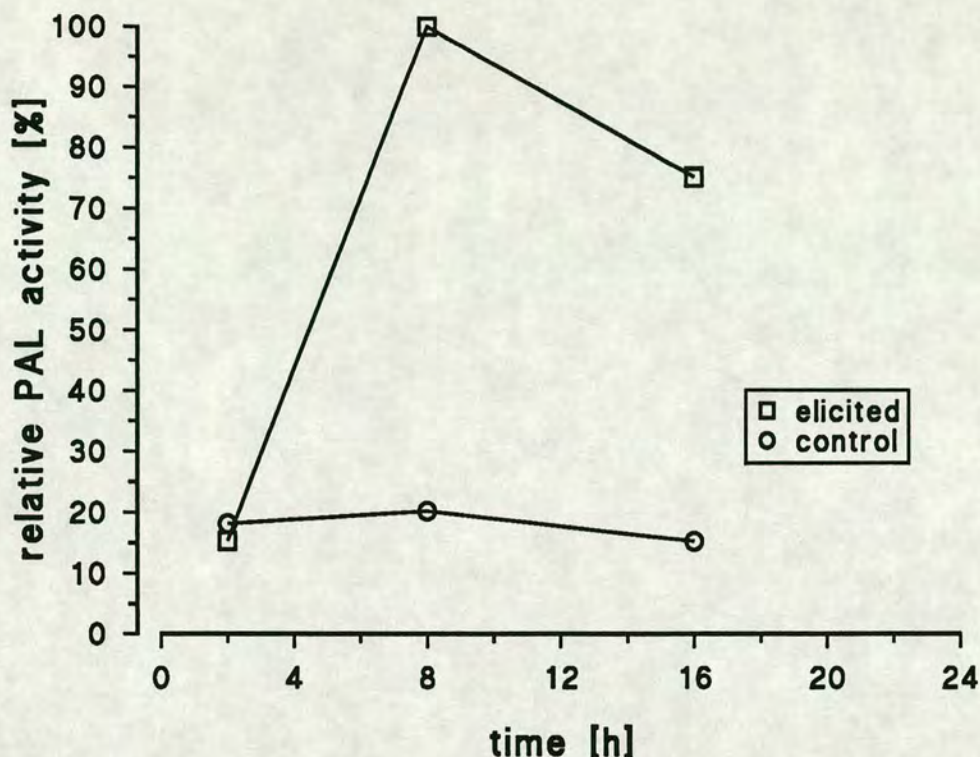


Figure 3.4.1 PAL activities of elicited and un-elicited *Capsicum* cell suspensions during the time course of elicitation. Activities are expressed relative to the highest specific activity measured. Three replicates per data point.

3.4.2 Ammonium sulphate precipitation of PAL activity

In an approach to concentrate PAL activity, replicates of crude enzyme extracts were combined and the protein precipitated by the addition of ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$).

Enzyme extracts were adjusted to the desired ammonium sulphate concentration by adding small quantities of solid $(\text{NH}_4)_2\text{SO}_4$ while stirring vigorously at 4°C (Schleif and Wensink, 1981). Protein was precipitated for 20 min at 40% $(\text{NH}_4)_2\text{SO}_4$ saturation and pelleted for 20 min at 12000xg. The supernatant was adjusted to 55% $(\text{NH}_4)_2\text{SO}_4$ saturation and protein was precipitated and pelleted as above. Precipitated proteins were resuspended in 0.2 M Tris-HCl (pH 8.8) and thoroughly dialysed against the same buffer. Specific PAL activity was determined as described in section 2.5.3.

PAL activity was precipitated in the protein fraction at 40-55% ammonium sulphate saturation.

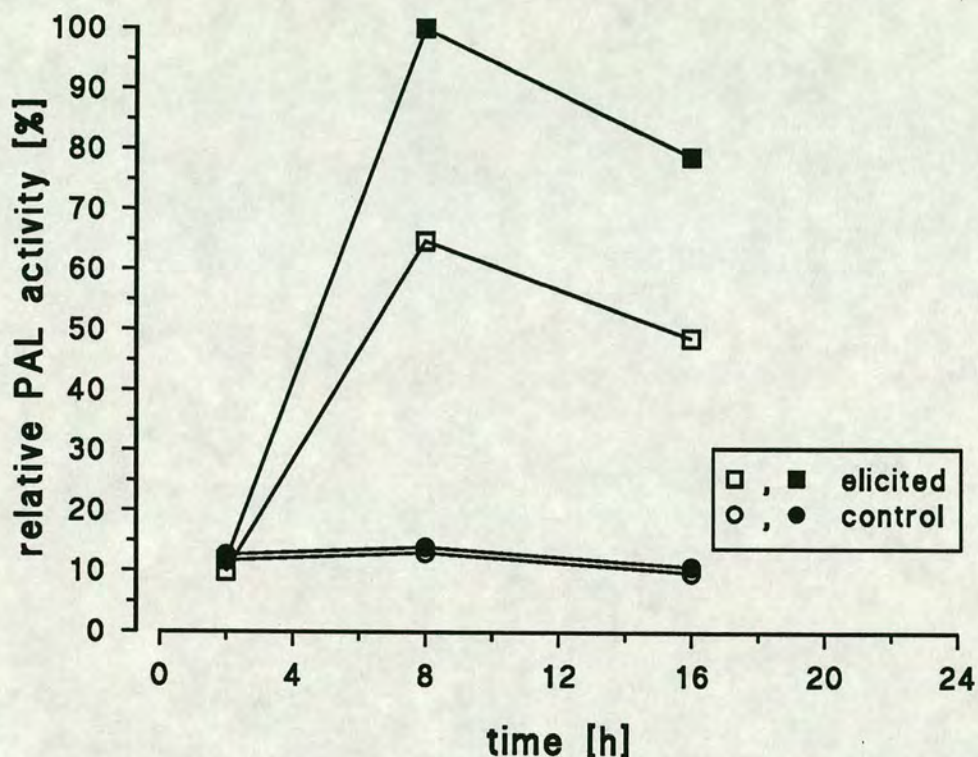


Figure 3.4.2 PAL activities of crude enzyme extracts and 40-55% $(\text{NH}_4)_2\text{SO}_4$ cuts. Open symbols represent measurements from crude extracts, solid symbols represent measurements from ammonium sulphate cuts. Activities are expressed relative to the highest PAL activity measured.

In Fig. 3.4.2 the specific PAL activities of crude enzyme extracts and 40-55% $(\text{NH}_4)_2\text{SO}_4$ cuts are plotted against elicitation time. The pattern of induction of PAL activity was not influenced by ammonium sulphate fractionation. The levels of specific PAL activity in the $(\text{NH}_4)_2\text{SO}_4$ cuts were elevated compared to the measurements in crude enzyme extracts. Specific activities measured in $(\text{NH}_4)_2\text{SO}_4$ fractions were 1.2-1.6 times the activity measured in crude extracts. However, detailed investigation of various $(\text{NH}_4)_2\text{SO}_4$ cuts showed that losses in total PAL activity were

considerable. Precipitation of protein in 80% ammonium sulphate saturation resulted in a recovery of only 25% of the total PAL activity. Concentration of PAL activity up to 1.6 times the value for crude extracts as presented in Fig. 3.4.2 resulted from a reduction in protein content that was greater than the loss in total PAL activity.

PAL protein from other plant sources is known to become degraded during purification (Bolwell *et al.*, 1986a; Schröder *et al.*, 1976).

Crude methods, like ammonium sulphate precipitation, are commonly used as a first measure to reduce bulk amounts of protein in enzyme samples. Ammonium sulphate fractionation was chosen to concentrate PAL activity because it is relatively fast and easy to perform. However, due to the small increase in specific PAL activity achieved with this method and the great losses of total activity during the concentration procedure, ammonium sulphate cuts were considered to offer little advantage over the crude enzyme preparations. Consequently, crude enzyme extracts were used for all further studies on PAL activity and PAL protein sub-units.

3.4.3 Early stages of PAL activity induction following elicitation of *Capsicum* cultures

The results of the experiments described in section 3.4.1 demonstrated that treatment of *Capsicum frutescens* cell cultures with fungal elicitor released from spores of *Gliocladium deliquescens* leads to an increase in PAL activity as determined by an *in vitro* PAL assay. Treatment of control cultures with an equivalent volume of water was used to ensure the detection of possible dilution effects on PAL activity. Un-elicited cultures showed low basal levels of specific PAL activity throughout the elicitation period. It can be concluded, that the volume of the fungal elicitor (1-1.5 ml) is too small to cause any significant dilution effects on PAL activity when added to 50 ml suspension cultures. The observed induction of PAL activity in elicited cultures was therefore caused by the presence of fungal elicitor alone. Thus, it was not necessary to take control samples at every time point during elicitation experiments.

The following experiments were designed to study in more detail the induction of PAL activity during elicitation of stationary-phase *Capsicum* suspension cultures. Attention was focussed on the early part of the time course, in anticipation of the PAL mRNA investigations to follow, and a putative *de novo* synthesis of the enzyme during elicitation.

PAL activities were followed for 24 h after inoculation of the cultures with the fungal elicitor preparation. The results are presented in Table 3.4.2 and Fig. 3.4.3.

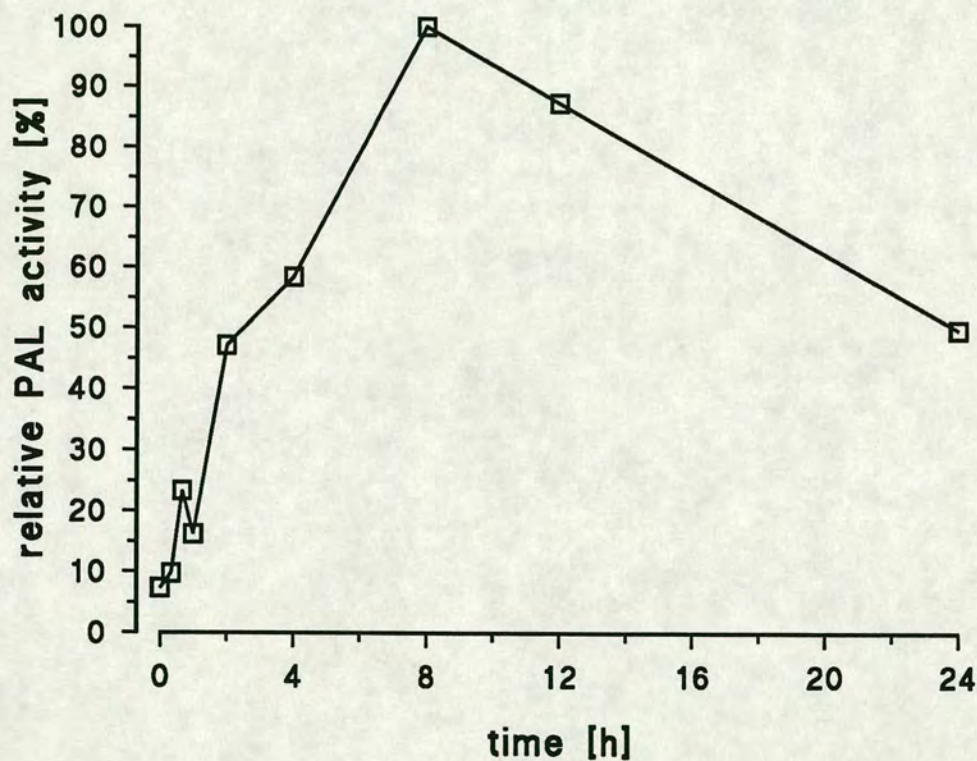


Figure 3.4.3 Elicitation time course experiment paying particular attention to the early stages after the onset of the elicitor treatment. PAL activities were plotted relative to the highest activity measured.

Table 3.4.2 Specific PAL activities extracted from *Cap-sicum frutescens* cell suspension cultures following treat-ment with a fungal elicitor preparation. Data represent arithmetic means of three replicates \pm standard error.

	specific PAL activity [μ kat·(kg protein) ⁻¹]		
control	4.05	\pm	2.9
20 min	5.40	\pm	1.1
40 min	12.90	\pm	0.4
1 h	9.00	\pm	0.7
2 h	26.00	\pm	0.7
4 h	32.20	\pm	4.9
8 h	55.20	\pm	6.2
12 h	48.10	\pm	2.5
24 h	27.50	\pm	0.7

PAL activities at t_0 were determined from un-elicited cultures. Controls at later time points were not taken because, as shown in Fig. 3.4.1, PAL activities of control cultures did not change throughout the elicitation time course.

A small increase in PAL activity was detected as early as 20 min after the onset of the elicitor treatment. However, PAL activities at t_0 and $t_{20\text{min}}$ were not significantly different as estimated by "Student's t" analysis. The first PAL activity significantly different (5% level) from the initial activity was measured at $t_{40\text{min}}$. This was followed by a slight drop in PAL activity at $t_{1\text{h}}$. Statistical analysis showed that the decrease in PAL activity at $t_{1\text{h}}$ was not significant compared to the specific activity measured at $t_{40\text{min}}$. The sample means at $t_{40\text{min}}$ and $t_{1\text{h}}$ were not significantly different. PAL activity increased sharply between 1 h and 2 h after the addition of the fungal elicitor preparation. Specific PAL activity measured at $t_{2\text{h}}$ was significantly different (1% level) from the previous measurements at t_0 - $t_{1\text{h}}$.

This observation did not correspond to the results obtained for PAL activity measurements in section 3.4.1 where no significant difference could be detected between the controls and 2 h elicited cultures. It should be noted, that for the preliminary studies in section 3.4.1 *Capsicum* cell cultures of a different developmental stage had been used.

PAL activity increased steadily and reached its highest value after 8 h. However, PAL activities at t_{8h} and t_{12h} were not significantly different. This might be partly due to the relatively large standard deviation of the sample mean at t_{8h} , but there is a possibility that PAL activity has a maximum not at t_{8h} but somewhere between t_{8h} and t_{12h} .

3.4.4 Summary

The experiments in section 3.4 were designed to determine the pattern of induction of specific PAL activity in *Capsicum frutescens* cell cultures during elicitation. The preliminary study in section 3.4.1 demonstrated that during the time course of elicitation PAL activity in control cultures remained unchanged. It can also be concluded that the addition of 1-1.5 ml elicitor preparation was not enough to cause any dilution effect on PAL activity. The induction of PAL activity in elicited cultures was therefore caused by the presence of the fungal elicitor alone since controls were treated as elicited cultures in all other respects.

Ammonium sulphate fractionation was used in section 3.4.2 as an approach to concentrate PAL activity from crude enzyme extracts. A concentration of up to 1.6 fold was achieved but the losses in total activity were considered not tolerable for use as a routine method. The detailed investigation of PAL activity induction described in section 3.4.3 was consequently conducted using crude enzyme extracts. The attention in this study was focussed on the early part of the time course of elicitation. In contrast to the preliminary study in section 3.4.1 the detailed time course showed induction of PAL activity 40 min after the onset of elicitation. Maximum PAL activity was detected approximately 8 h after elicitation. However, the data indicate that the true maximum PAL activity could be nearer to t_{10h} .

The nature of the established induction of PAL activity following elicitation of *Capsicum frutescens* cell cultures will now be investigated further at the translational level using *in vivo* labelling techniques to discover whether there is *de novo* synthesis of the enzyme.

3.5 Investigation of PAL protein synthesis during elicitation of *Capsicum* suspension cultures

The experiments described in section 3.4 showed the pattern of induction of PAL activity during elicitation of *Capsicum frutescens* cell cultures. Extractable specific PAL activity increased sharply in elicited cultures and did not change in control cultures. The earliest induction of PAL activity was detected 40 min after the addition of the fungal elicitor. Maximum specific activity was reached 8-10 h after elicitation and decreased slowly thereafter. In this section the induction of PAL activity will be investigated at the translational level using *in vivo* labelling techniques to discover whether there is *de novo* synthesis of the enzyme.

3.5.1 Identification of PAL protein sub-units

The study of PAL induction at the translational level required the identification of the enzyme sub-units.

The identification of PAL protein was possible without extensive purification procedures by the use of polyclonal antibodies raised against PAL protein from other plant sources. Rabbit immune serum containing antibodies raised against parsley PAL protein was a kind gift from Prof. K. Hahlbrock (MPI für Züchtungsforschung, Cologne, Germany).

Two methods were used with the anti-(parsley PAL) serum to identify PAL protein, direct immunoprecipitation and immunoblotting.

3.5.1.1 Immunoprecipitation of PAL protein

Direct immunoprecipitation is one of the earliest and simplest immunochemical techniques (Harlow and Lane, 1988). The antigen-antibody complex formed can (directly) be removed from the solution by centrifugation without the use of anti-immunoglobulin antibodies, provided the complex is sufficiently large.

Immunoprecipitation was performed as described by Schröder *et al.* (1976). A portion (1 ml) of crude enzyme extract, prepared as described in section 2.5.1, was mixed with 15 µl anti-(parsley PAL) serum. The mixture was incubated for 30 min at room temperature, left for 18 h at 4°C, and centrifuged for 5 min in an Eppendorf desk-top centrifuge. The pellet was washed three times with 0.2 ml ice-cold 0.15 M NaCl and dissolved in 0.1 ml SDS-PAGE sample buffer (section 2.8.2). Proteins were size separated by SDS-PAGE (section 2.8). PAL protein sub-units had to be identified by immunochemical detection because the antigen was not radioactively labelled. Therefore, the size separated proteins were transferred to nitrocellulose by Western Blotting (section 2.9). PAL protein fixed to the membrane was hybridised with primary antibody (rabbit anti-PAL) and colloidal gold labelled secondary antibody (goat anti-rabbit) as described in section 2.10. Enhancement of the pink coloured signal was achieved by the precipitation of metallic silver on the colloidal gold particles (section 2.10) resulting in a high-contrast dark brown to black signal (Fig. 3.5.1).

However, Western Blots of immunoprecipitated PAL protein (Fig. 3.5.1) showed a number of bands with major signals relating to polypeptides approximately 81 and 57 kD in size. The identity of these immunoprecipitated polypeptides is not clear since PAL from other plant sources is reported to be a tetrameric enzyme consisting of identical sub-units. Reported values of the molecular size of the native enzyme sub-units range from 83 kD to 55 kD (Schröder *et al.*, 1976; Bolwell *et al.*, 1985; Gupta and Acton, 1979). However, suggestions have been made concerning the possible ease of partial degradation of the enzyme *in vitro* and *in vivo* (Bolwell *et al.*, 1985; 1986a). Furthermore, anti-(parsley PAL) serum was found not to be monospecific for the enzyme at least from some sources and coprecipitation of unrelated polypeptides has been reported (Bolwell *et al.*, 1985).

The results obtained from immunoprecipitations using anti-(parsley PAL) serum (Fig. 3.5.1) did not allow the conclusive identification of PAL protein sub-units. The reasons for the observed coprecipitations could be partial degradation of the antigen, lack of mono-specificity of the antibody, or coprecipitation, due to the size of the antigen-antibody complex. Considering the relatively large volume of antiserum required for each immunoprecipitation and its limited supply, immunoprecipitations were not pursued as an approach to identify *Capsicum* PAL protein.

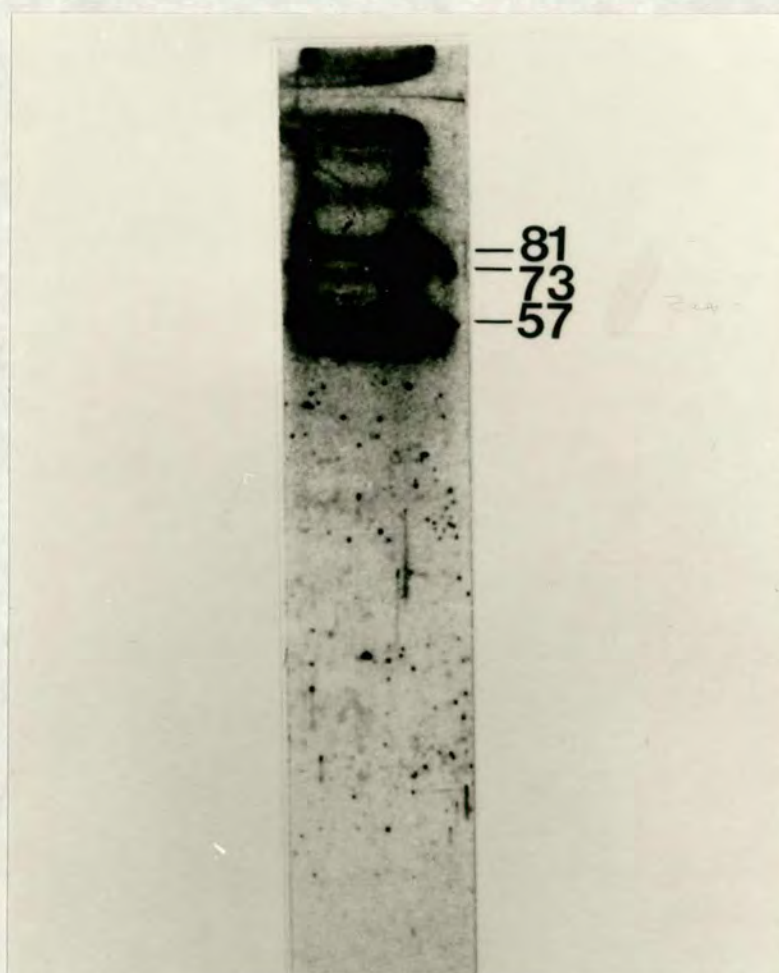


Figure 3.5.1 Immunoprecipitation of *Capsicum frutescens* PAL protein. The immunoprecipitate was size separated by SDS-PAGE and subjected to Western Blotting.

3.5.1.2 Detection of PAL protein by immunoblotting

Immunoblotting is useful when dealing with antigens that are easily degraded and thus, less amenable to analysis by immunoprecipitation (Harlow and Lane, 1988).

Immunoblotting was performed using crude enzyme extracts without concentrating the antigen by immunoprecipitation. Protein from crude enzyme preparations (section 2.5.1) was size separated by SDS-PAGE (section 2.8) and transferred to nitrocellulose membrane (section 2.9). Immunochemical detection of PAL protein was performed as described in section 2.10. Hybridisation solutions containing antibodies were re-used many times without apparent loss of activity.

At first, the immunoblots obtained had high background staining and showed a high degree of unspecific hybridisation (data not shown). Careful refinement of the technique included alterations in the stringency of washings, antibody concentrations, hybridisation times, and a comparison of various blocking reagents. The immunochemical detection method used as routine is described in section 2.10.

Immunoblotting of enzyme preparations from elicited *Capsicum* cultures resulted in two major bands of approximately 77 kD and 70 kD (Fig. 3.5.2, lane C). Immunoblotting of proteins from un-elicited (control) *Capsicum* suspension cultures is shown in lane B (Fig. 3.5.2). In enzyme preparations from elicited and control cultures a 70 kD protein band was detected immunochemically. The signal was of similar intensity in both enzyme preparations as judged visually. Immunoblots from elicited samples and controls differed in the detection of the 77 kD protein band. A strong signal was obtained for elicited samples and no corresponding signal could be detected in immunoblots from enzyme preparations of control cultures.

In order to confirm that the 77 kD protein detected in elicited cultures corresponded to native PAL protein sub-units immunoblotting was performed using enzyme preparations obtained from parsley leaves (Fig. 3.5.2, lane A). Crude enzyme

extracts from parsley leaves were prepared using the method for PAL extraction from *Capsicum* cells (section 2.5.1).

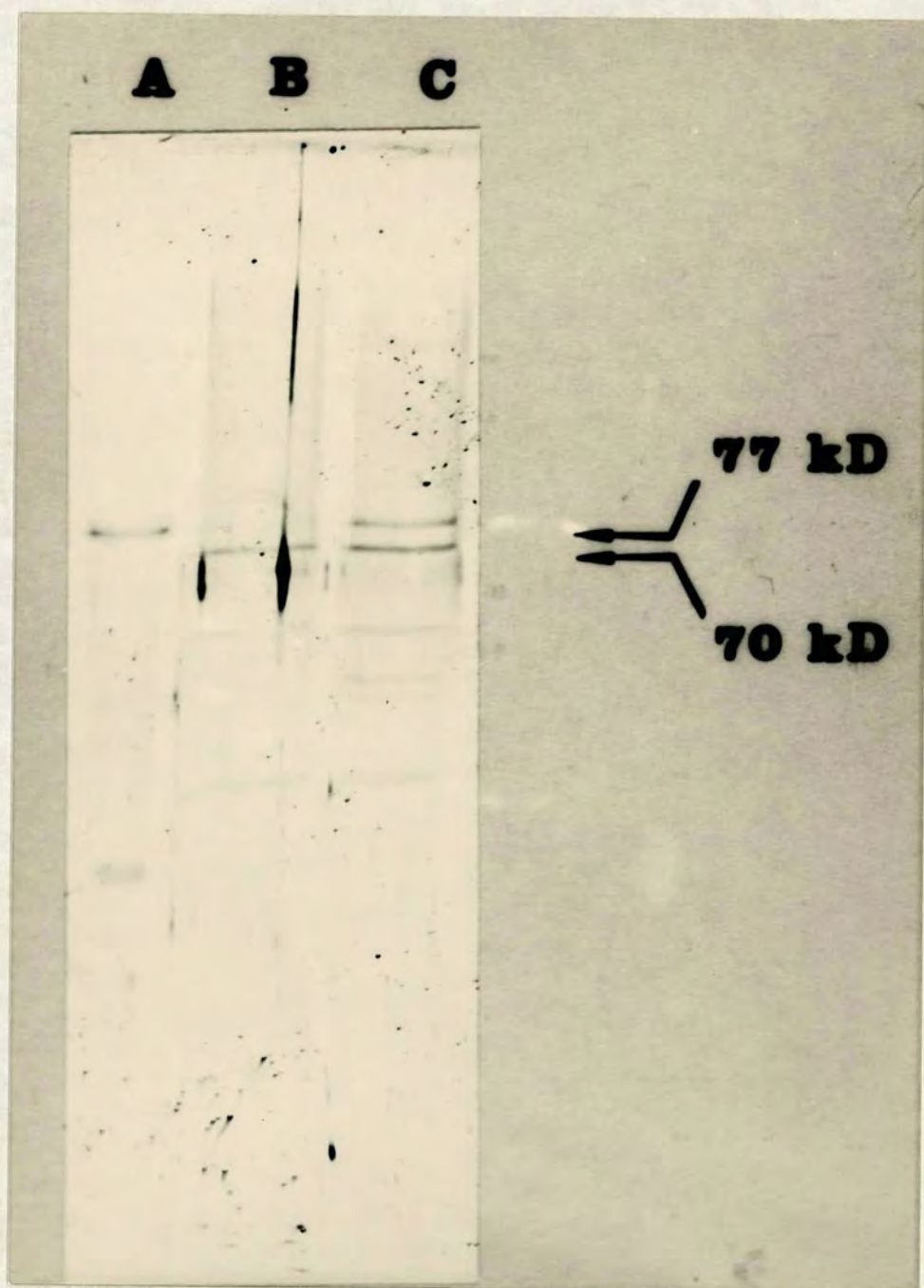


Figure 3.5.2 Immunoblotting of crude enzyme preparations obtained from (A) parsley leaves, (B) untreated *Capsicum frutescens* suspension cultures, (C) elicited *Capsicum frutescens* suspension cultures. Hybridisation of antigen with rabbit anti-(parsley PAL) serum was visualised using colloidal gold labelled goat anti-rabbit (GAR) immunoglobulins and by silver enhancement of the colloidal gold particles.

Immunoblots of crude enzyme preparations from parsley leaves (Fig. 3.5.2, lane A) using anti-(parsley PAL) serum resulted in one strong signal at *ca.* 77 kD which represented parsley PAL protein sub-units.

The mobility of the 77 kD polypeptide detected by anti-(parsley PAL) serum in samples from elicited *Capsicum* cultures was identical with PAL protein sub-units from parsley. This polypeptide was only present in *Capsicum* cultures when PAL activity was induced by elicitor treatment. It could not be detected in control cultures. PAL sub-units from various plant species are often characterised as 77 kD in size (Bolwell *et al.*, 1986a,b; Lawton *et al.*, 1983a). Taken together, the data indicate that the polypeptide with an apparent molecular weight of 77 kD, immunochemically detected from extracts of elicitor-treated *Capsicum* cells by anti-(parsley PAL) serum, is the corresponding enzyme sub-unit from chilli pepper.

The same amount of protein (5 µg) was loaded per lane for all immunoblots. The 77 kD sub-unit was not detectable as a band in *Capsicum* extracts when polyacrylamide gels were stained with Coomassie Brilliant Blue (data not shown). No statistically significant difference in total amounts of soluble protein could be detected between elicited *Capsicum* cultures and controls. However, treatment with fungal elicitor can alter the total protein composition considerably (Bollmann and Hahlbrock, 1990). The strong signal at 70 kD, present in immunoblots of crude enzyme extracts from elicited and control cultures of *Capsicum frutescens*, was not detected in immunoblots of enzyme extracts from parsley leaves. The 70 kD signal was of similar intensity in elicited samples and controls and seemed not to be induced during elicitation. Although, the 70 kD polypeptide was recognised by anti-(PAL) serum the evidence presented here is not conclusive as to whether or not the polypeptide is related to *Capsicum* PAL. The identity of the 70 kD protein will be discussed in detail in chapter four of this thesis.

Capsicum PAL sub-units were identified by immunoblotting techniques and their molecular size was estimated. PAL sub-units extracted from parsley leaves (Fig. 3.5.2, lane A) and elicited *Capsicum* cultures (Fig. 3.5.2, lane C) had similar electrophoretic mobility in polyacrylamide gels. Their molecular size was estimated to be 77 kD by their relative positions to molecular weight marker proteins run alongside the samples on the same gel. The molecular size of *Capsicum* PAL seems to correspond to the size reported for the enzyme from bean and parsley (Bolwell *et al.*, 1986a,b).

In Fig. 3.5.2 the same amount of protein (5 µg) was loaded onto the gel for all samples. However, PAL activities calculated for the blotted samples were not the same due to different specific enzyme activities. Extracts from parsley leaves and *Capsicum* control cultures containing 5 µg protein had similar PAL activities, 0.15 and 0.26 pkat respectively. Samples (5 µg protein) from elicitor-treated *Capsicum* suspension cultures contained *ca.* 1.43 pkat PAL activity. Therefore, a much stronger signal for elicited *Capsicum* samples was expected compared to parsley samples, provided that parsley PAL and *Capsicum* PAL have similar numbers of sub-units and active sites. The signals obtained for parsley PAL and *Capsicum* PAL were of similar intensity despite the fact that the (elicited) *Capsicum* samples contained *ca.* 9.5 times the enzyme activity calculated for the parsley samples. Although, parsley samples and samples from *Capsicum* control cultures had comparable PAL activities no 77 kD signal was obtained for immunoblots of *Capsicum* controls. It can be concluded, that hybridisation of *Capsicum* PAL with anti-(parsley PAL) serum results in a much weaker signal than the one obtained by hybridisation of comparable activities of parsley PAL with the same anti-serum. Furthermore, only steady-state levels could be determined by immunoblotting because the antigen was not radioactively labelled. Immunoblotting as described above was therefore not considered a suitable method for determining whether *de novo* synthesis of PAL protein occurs during elicitation of *Capsicum frutescens* suspension cultures. However, the technique allowed the

identification of *Capsicum* PAL sub-units and the subsequent determination of their electrophoretic mobility which formed the basis for the detection of newly synthesised enzyme sub-units by *in vivo* labelling of protein synthesised following elicitor treatment of *Capsicum frutescens* cell suspensions.

3.5.2 Protein synthesised during elicitation of *Capsicum frutescens* cell cultures

3.5.2.1 Continuous labelling of protein synthesised

Protein synthesised during elicitation of *Capsicum frutescens* suspension cultures was labelled using a mixture of [³⁵S]methionine and [³⁵S]cysteine (section 2.7.1). Radiolabel equivalent to 3.7 MBq [³⁵S]methionine and the fungal elicitor preparation (section 2.4.1) were added simultaneously to *Capsicum* cell suspensions (50 ml per experimental flask). Elicitation and continuous *in vivo* labelling of protein synthesis was carried out for 4 h, 8 h, 12 h, and 24 h. Protein in control cultures was labelled for 24 h in the absence of fungal elicitor. Cells were harvested by vacuum filtration and PAL activity was extracted as described in section 2.5. Crude enzyme extracts were subjected to SDS-PAGE (section 2.8) and radiolabel in size separated proteins was detected by fluorography (section 2.11.2).

Approximately 10% of the activity of labelled amino acids added was associated with synthesised protein at the end of the experiment. It was necessary to use an excess amount of radiolabel to prevent exhaustion of the label during the course of the experiment.

The amount of soluble protein extracted from *Capsicum frutescens* cells was similar for all samples. Protein content per g FW was estimated from protein assays (section 2.6) using crude enzyme extracts. It was found that samples from elicited and control cultures contained *ca.* 2 mg soluble protein per g FW (Table 3.5.1). Although, the total amount of protein was not changed by elicitation of *Capsicum* cell cultures

the rate of protein synthesis was transiently increased as estimated by the total amount of label incorporated into protein (Table 3.5.2 and Fig. 3.5.3).

Table 3.5.1 Soluble protein content of *Capsicum* cells was determined from crude enzyme preparations and estimated per g FW by extrapolation. Data represent arithmetic means of three replicates \pm standard error.

sample	mg protein·(g FW) ⁻¹ \pm SE		
control	1.892	\pm	0.006
4 h elicited	1.927	\pm	0.002
8 h elicited	1.876	\pm	0.000
12 h elicited	1.930	\pm	0.006
24 h elicited	1.904	\pm	0.004

Table 3.5.2 The intensity (blackening) of protein bands on the fluorograph (Fig. 3.5.4) was quantified by densitometer scans (Fig. 3.5.6). The data represent the peak areas (relative units) for ³PAL protein, an induced 32 kD polypeptide, and the sum of all peak areas per lane (total).

	³ PAL ^a (% of total)		32 kD (% of total)		total
control	3835.6	(10.8)	8924.7	(25.3)	35230.2
4 h	26977.4	(19.0)	7785.9	(5.5)	141292.6
8 h	18795.6	(21.2)	15443.6	(17.4)	88623.5
12 h	13887.8	(20.5)	7762.7	(11.4)	67583.8
24 h	7136.7	(18.9)	7177.5	(19.0)	37758.8

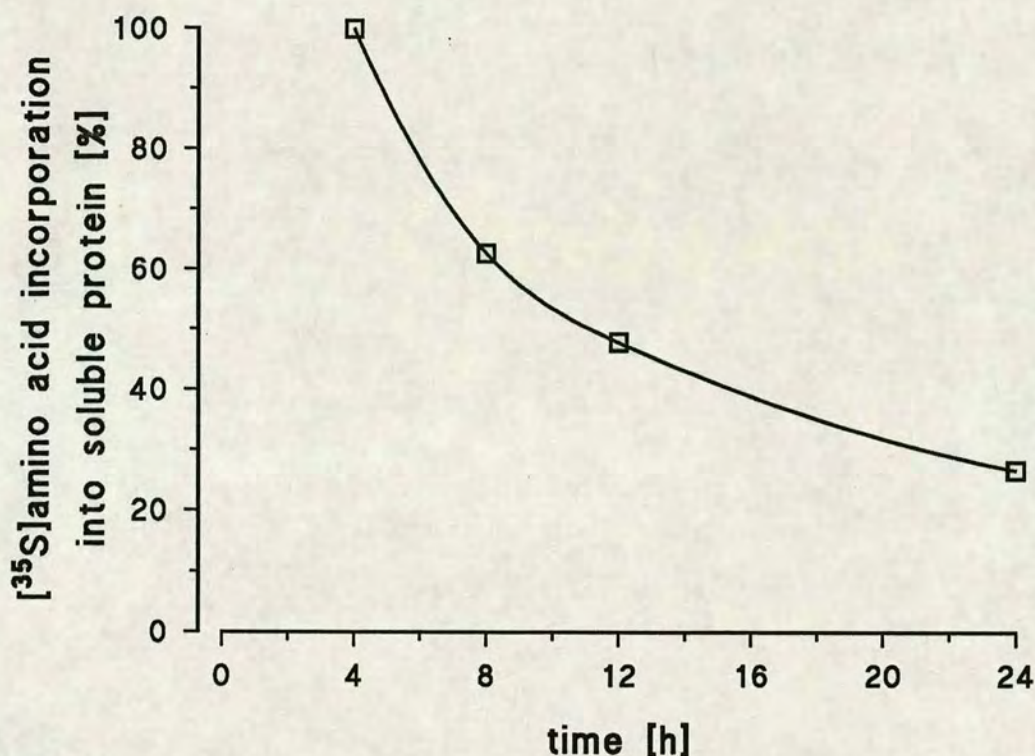


Figure 3.5.3 The total amount of labelled amino acids incorporated into protein was estimated by densitometer scans of fluorographs of polyacrylamide gels. Incorporation of radiolabel was expressed relative to the highest value observed.

Treatment of *Capsicum frutescens* suspension cultures with a fungal elicitor preparation changed the total protein composition as shown in Fig. 3.5.4. The most notable change occurred in a protein band representing a polypeptide of *ca.* 77 kD. Its molecular size was estimated by interpolation from its relative mobility compared to the relative mobility of marker proteins run on the same gel (Fig. 3.5.5).

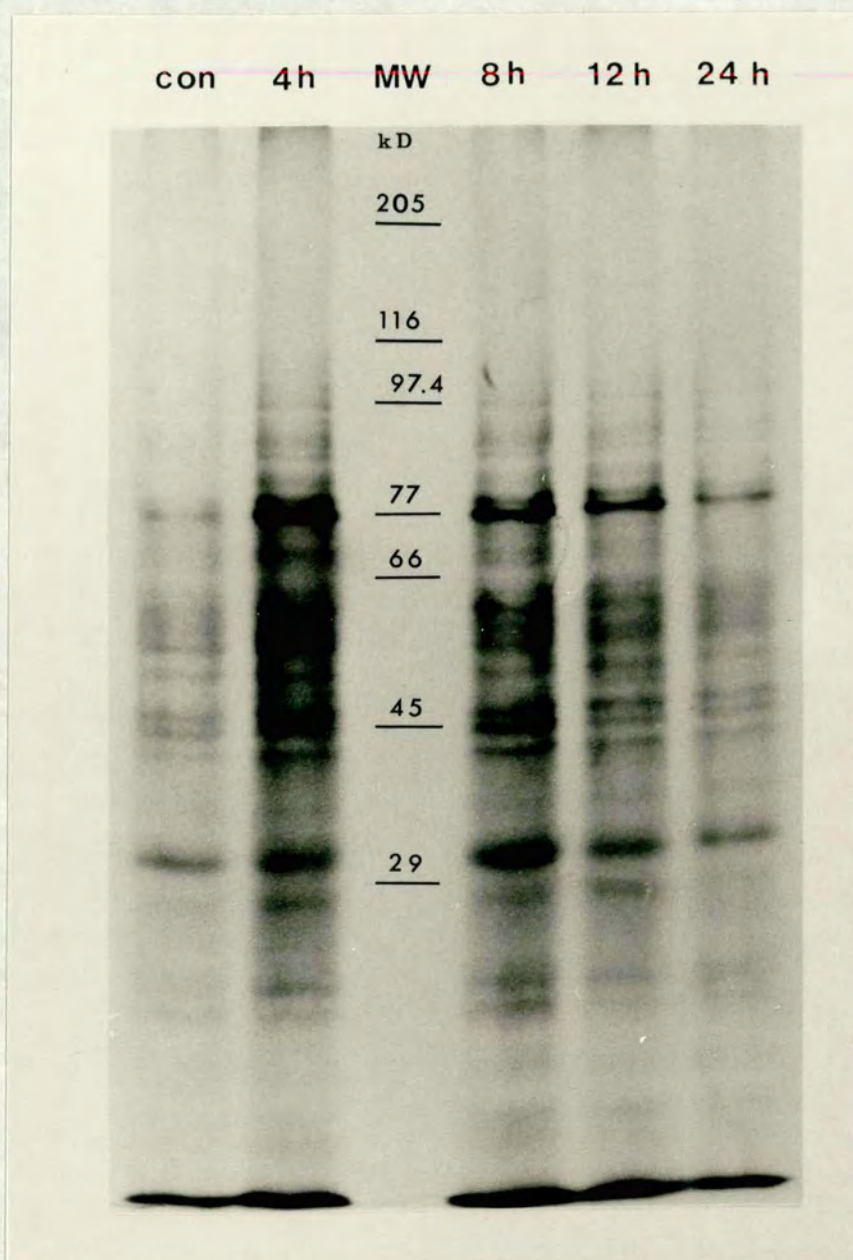


Figure 3.5.4 Protein in crude enzyme preparations was size separated by SDS-PAGE and labelled amino acids were detected by fluorography. Control cultures (con) were labelled for 24 h in the absence of fungal elicitor. Molecular weight marker proteins (MW) were non-radioactive, their positions and molecular sizes are indicated. Samples obtained from elicited cultures are marked with their corresponding elicitation time (4 h, 8 h, 12 h, 24 h).

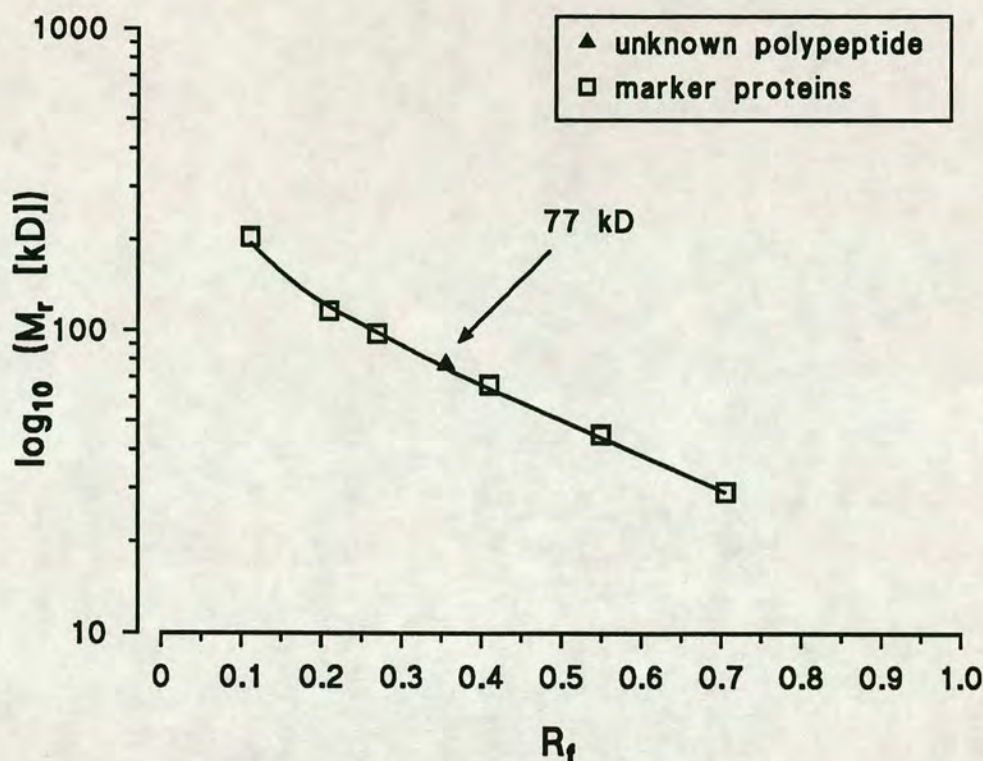


Figure 3.5.5 Estimation of the molecular size of a polypeptide synthesised following treatment of *Capsicum frutescens* cell cultures with a fungal elicitor preparation. The marker proteins were carbonic anhydrase (bovine erythrocytes, 29 kD), albumin (ovalbumin, 45 kD), albumin (bovine serum, 66 kD), phosphorylase B sub-unit (rabbit muscle, 97.4 kD), β -galactosidase sub-unit (*Escherichia coli*, 116 kD), myosin sub-unit (rabbit muscle, 205 kD).

The molecular size (77 kD) estimated for the newly synthesised polypeptide corresponded to the molecular size for the *Capsicum* PAL protein sub-units identified in section 3.5.1. Radioactivity associated with the 77 kD protein in control samples contributed *ca.* 10% of the total activity associated with protein in crude enzyme extracts (Table 3.5.2). This ratio increased to 20% following elicitor treatment (Table 3.5.2 and Fig. 3.5.6) ^{with} consistent the induction of *de novo* synthesis of this polypeptide. The high proportion (10-20%) of the 77 kD protein in crude enzyme extracts corresponds to the fact that crude enzyme preparations were obtained by a method optimised for the extraction of PAL activity. Taken together, it can be concluded that the observed 77 kD protein (Fig. 3.5.4) might be PAL and hereafter will be referred to as "PAL".

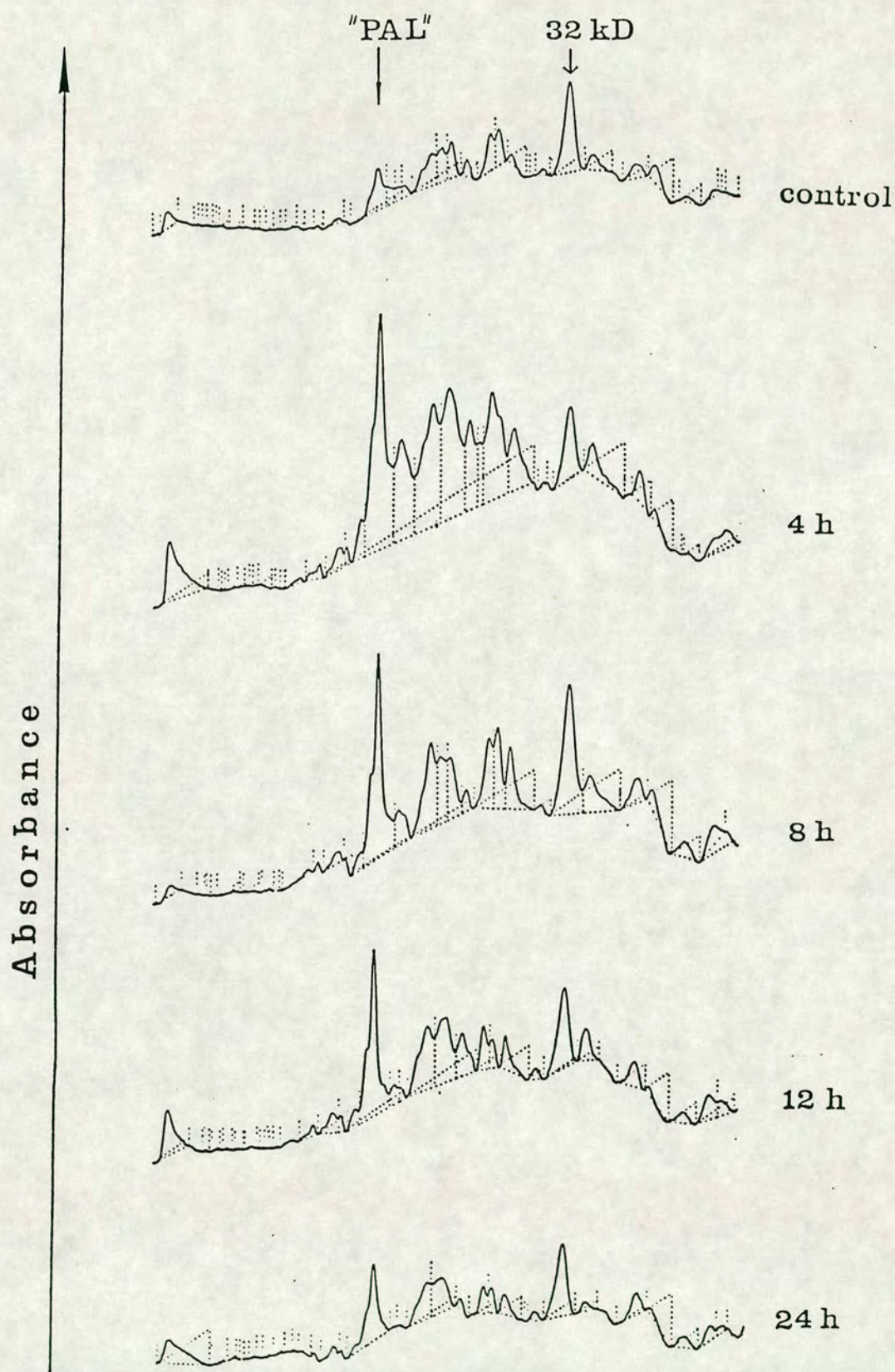


Figure 3.5.6 Fluorographs (Fig. 3.5.4) were analysed by densitometer scanning. Intensities of protein bands (blackening of the film) were estimated by their absorbances at 540 nm wavelength. The peak areas were quantified in relative units (Table 3.5.2).

The detailed comparison of the incorporation of labelled amino acids into "PAL" protein is shown in Fig. 3.5.7. The incorporation of radioactivity into "PAL" protein followed a similar pattern to the incorporation of radiolabel into total protein (Fig. 3.5.3).

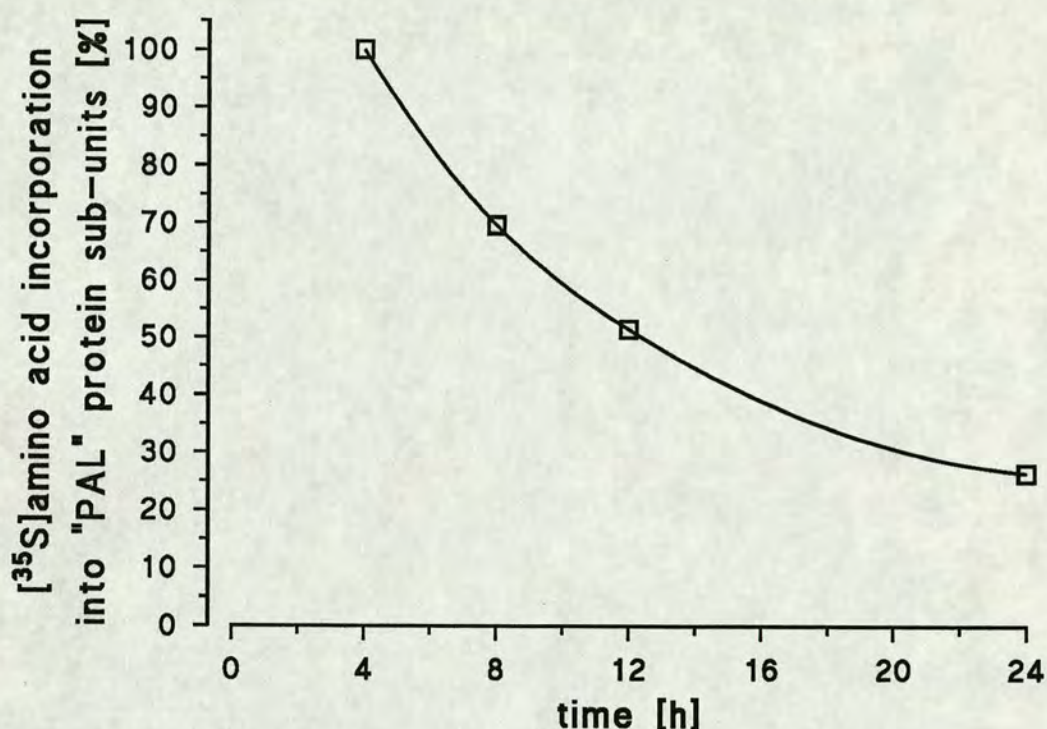


Figure 3.5.7 The incorporation of [³⁵S]amino acids into "PAL" protein sub-units was deduced from densitometer scans and plotted relative to the highest value observed.

From the data presented it can be concluded, that the observed induction of "PAL" protein synthesis (Fig. 3.5.7) was the result of a combination of a general induction of protein synthesis (Fig. 3.5.3) and a specific induction of "PAL" protein synthesis. The latter increased the relative amount of "PAL" protein in crude enzyme extracts from *ca.* 10% in controls to *ca.* 20% in elicited samples. However, the observed induction of protein synthesis during elicitation was too small to change the absolute amounts of total protein (Table 3.5.1) in crude enzyme extracts. Consequently, the same volume

of crude enzyme extract loaded onto polyacrylamide gels resulted in similar intensities of Coomassie protein stain (data not shown). Although, "PAL" protein contributed 10-20% of the labelled protein in PAL extracts its proportion of total (labelled and unlabelled) protein is much lower since the 77 kD band could not be detected by Coomassie staining. The detection limit of Coomassie Brilliant Blue for the described procedure (section 2.8.4) is 0.5-1.0 µg per protein band (Sigma, personal communication). It can be concluded, that "PAL" contributed less than 3% to the total amount of protein in crude enzyme extracts. Indeed, Lawton *et al.* (1983b) reported that the rate of PAL synthesis in elicited bean cells accounted for an estimated 0.4% of the total protein synthesised.

The specific induction of the synthesis of a second polypeptide, other than "PAL", was observed during elicitation of *Capsicum frutescens* cell cultures. A 32 kD protein, synthesised at relatively high levels in control cultures (Fig. 3.5.6), showed a marked increase in incorporation of [³⁵S]amino acids after a lag phase of 4-8 h. The highest level of incorporation was detected 8 h after the introduction of the fungal elicitor (Fig. 3.5.4), at a time when the total incorporation rate was on the decline from its maximum detected after 4 h (Fig. 3.5.3). The observed effect was transient and radiolabel incorporation into the 32 kD protein returned to control levels after 12 h elicitation (Table 3.5.2 and Fig. 3.5.6).

3.5.2.2 Pulse labelling of protein synthesised

The induction of "PAL" protein synthesis during the early stages of elicitor treatment of *Capsicum* cell cultures was studied by *in vivo* pulse labelling experiments. Experimental culture flasks were inoculated with a fungal elicitor preparation and 1.85 MBq [³⁵S]methionine was added 20 min before harvest of the cells. Elicitation times were 20 min, 40 min, 1 h, 2 h, 4 h, and 8 h. Controls received a 20 min pulse of [³⁵S]methionine in the absence of fungal elicitor. Samples of crude

enzyme extract containing 20 µg protein were subjected to SDS-PAGE (section 2.8). Size separated protein was transferred to nitrocellulose (section 2.9) and radioactivity was detected by direct autoradiography (section 2.11.1). [³⁵S]methionine was mainly incorporated into a 77 kD polypeptide ('PAL' sub-unit) during the 20 min pulse (Fig. 3.5.8) but some incorporation was also detectable in a number of bands ranging from 85 kD to 24 kD. These bands, detectable on the original autoradiographs but hardly visible on the photographic print, were indicated by arrows in Fig. 3.5.8. Control samples (Fig. 3.5.8, lane 0) did not show incorporation into 'PAL' protein but some incorporation was detected 20 min after introduction of the fungal elicitor. After 40 min the rate of 'PAL' synthesis had increased considerably as estimated by the incorporation of radiolabel into the 77 kD protein.

The rate of [³⁵S]methionine incorporation into 'PAL' protein during the 20 min pulses was approximately constant between 40 min and 4 h after the onset of elicitation and decreased thereafter, as estimated visually by the intensity of the 77 kD band on the autoradiographs.

The results obtained by pulse labelling experiments (Fig. 3.5.8) provided the necessary evidence for the specific increase in 'PAL' protein synthesis postulated from the results obtained by continuous labelling experiments (Fig. 3.5.4). The observed accumulation of radiolabel in 'PAL' protein during the first 4 h after introduction of the fungal elicitor was due to a high rate of *de novo* synthesis of the enzyme. The downturn of the rate of *de novo* synthesis 8 h after the introduction of the fungal elicitor (Fig. 3.5.8) resulted in a decrease in accumulation of labelled enzyme sub-units observed in continuous labelling experiments (Fig. 3.5.4) due to the turn-over of 'PAL' protein.

Elicitation of *Capsicum* cultures increased the rate of total protein synthesis (Fig. 3.5.3) not only the *de novo* synthesis of 'PAL' protein. Radioactive labelling of protein synthesis during a short pulse (20 min) was used to detect protein newly synthesised

with a high rate of translational activity. It can be concluded, that the synthesis of "PAL" protein was specifically induced during elicitor treatment resulting in predominantly labelled "PAL" protein sub-units following pulse labelling with [³⁵S]methionine (Fig. 3.5.8).

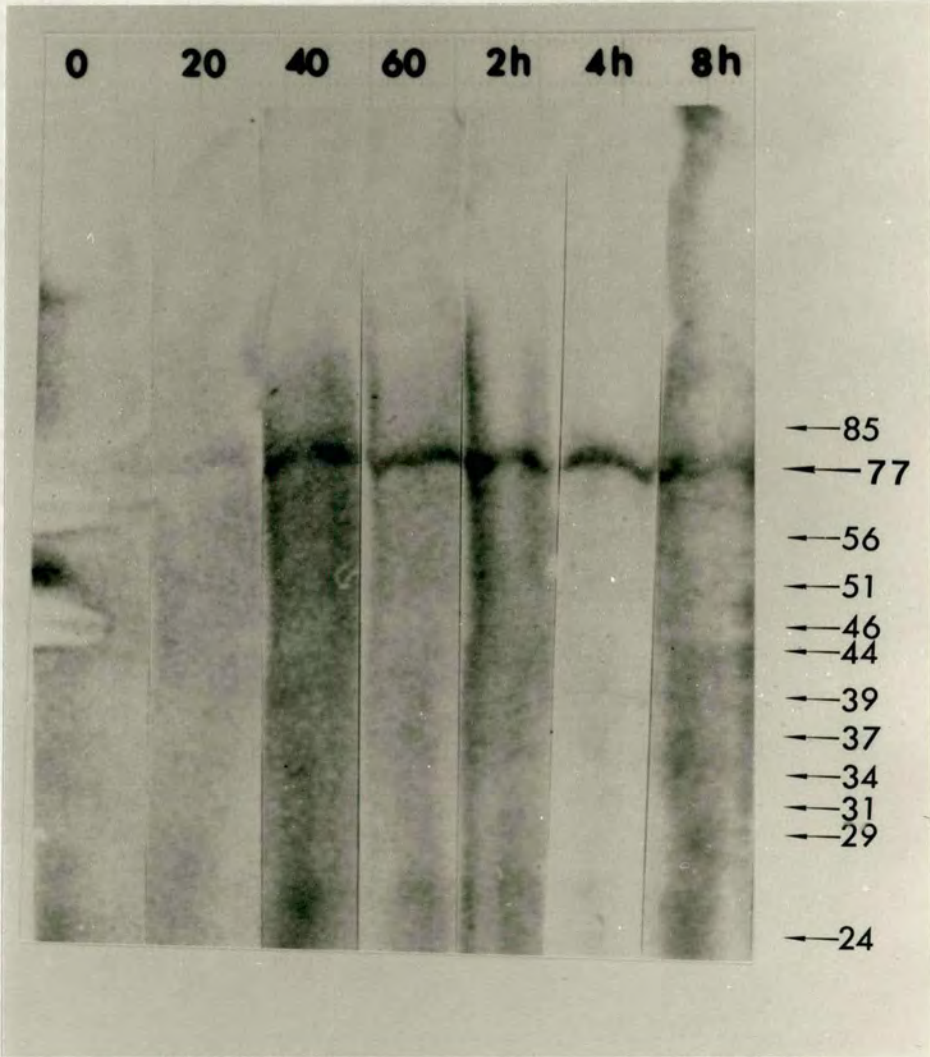


Figure 3.5.8 [³⁵S]methionine pulse labelling experiments. *Capsicum frutescens* suspension cultures were elicited for the time periods stated in the text and labelled with [³⁵S]methionine 20 min before harvest. Elicitation times are indicated above each lane. Controls (lane 0) were pulsed with [³⁵S]methionine in the absence of fungal elicitor. Arrows and numbers on the right hand side indicate bands detected and their corresponding molecular size.

3.5.3 PAL activity and "PAL" protein synthesis during elicitation of *Capsicum frutescens* cell cultures

The accumulation of [^{35}S]amino acids in "PAL" protein was chosen as an indicator for changes in the level of "PAL" protein present during elicitation of *Capsicum frutescens* cell cultures. It could be demonstrated, that the amount of "PAL" protein increased during elicitation up to 4 h after the introduction of the fungal elicitor and decreased thereafter. "PAL" protein levels were still elevated at the end of the experiment (24 h) compared to the levels measured in control cultures. However, the observed decline of "PAL" protein levels despite the continued presence of the fungal elicitor indicated that the effect of the elicitor on "PAL" protein synthesis was a transient one.

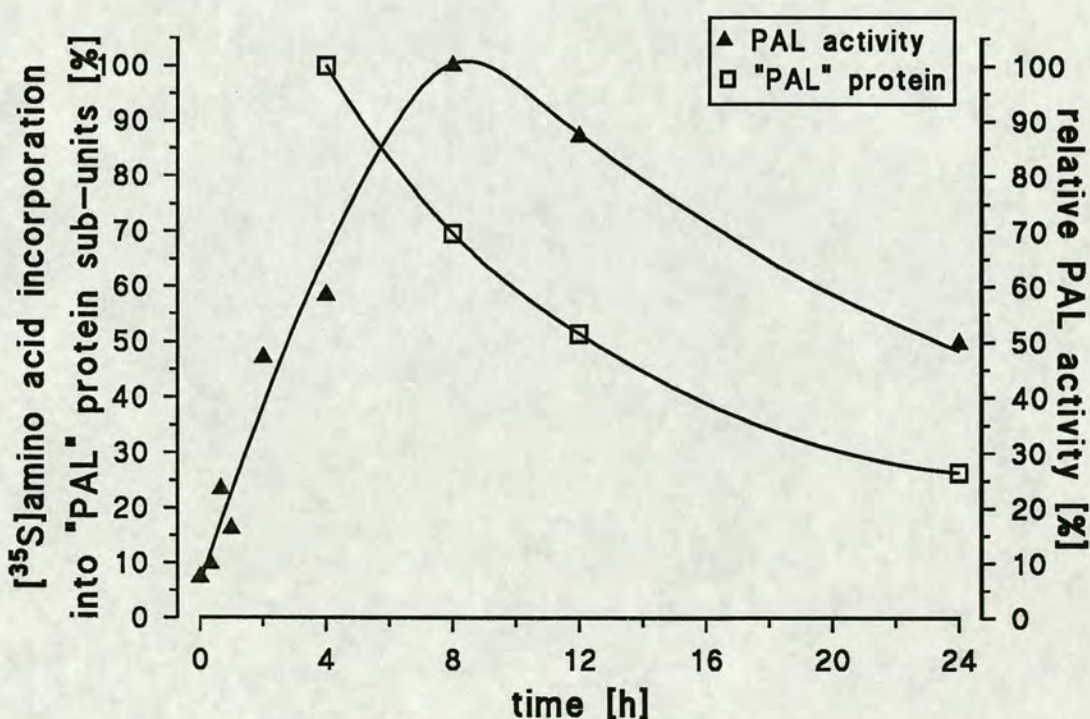


Figure 3.5.9 The incorporation of [^{35}S]amino acids into "PAL" protein sub-units was deduced from densitometer scans. Specific PAL activities were determined as described in section 3.4.1. Incorporation rate and PAL activity were expressed relative to the highest value observed.

Results from studies on extractable PAL activity following elicitation (section 3.4) revealed an induction pattern similar to the one observed for ³H-PAL protein synthesis. PAL activity increased during the early stages of elicitation, reached a maximum *ca.* 8 h after the introduction of the fungal elicitor, and decreased thereafter. The observed increase in PAL activity was preceded by an increase in newly synthesised ³H-PAL protein as shown in Fig. 3.5.9. Decreasing levels of ³H-PAL protein were followed by decreasing PAL activities. It can be concluded, that the observed induction of PAL activity, following elicitation of *Capsicum* cell cultures, was, at least in part, a consequence of the induction of *de novo* synthesis of the enzyme.

3.5.4 Summary

Protein synthesis during elicitation of *Capsicum frutescens* cell cultures was studied in section 3.5 and compared with observed changes in extractable PAL activity following elicitor treatment.

PAL protein was identified by immunoblotting and hybridisation with anti-(parsley PAL) serum. *Capsicum* PAL protein sub-units were found to have a similar electrophoretic mobility to parsley PAL sub-units and their molecular size was estimated as 77 kD. A 70 kD polypeptide was recognised by anti-(parsley PAL) serum in *Capsicum* samples and could not be detected in *Petroselinum* samples. This polypeptide was present in similar quantities in elicited and control cultures and thus, its synthesis was not induced during elicitation.

Immunoprecipitation proved unsuccessful in identifying PAL protein because it resulted in the coprecipitation of a number of polypeptides. A refinement of the technique was not attempted because of the relative large volume of anti-serum required for each immunoprecipitation and its limited supply.

Protein synthesis during elicitation of *Capsicum* cultures was followed using *in vivo* labelling techniques. Continuous labelling with [³⁵S]amino acids showed that total protein synthesis was increased following elicitation although, the increase was not sufficient to change the total amount of protein in crude enzyme extracts. An accumulation of radiolabelled "PAL" protein was found with a maximum 4 h after the onset of elicitation. Pulse labelling experiments using [³⁵S]methionine showed that "PAL" protein synthesis was transiently induced during elicitation. It was concluded that the combination of general induction of total protein synthesis and specific induction of "PAL" protein synthesis resulted in the observed accumulation of "PAL" protein during elicitation.

The synthesis of a 32 kD protein was transiently induced with a lag phase of 4-8 h. Maximum radiolabel incorporation was detected 8 h after elicitation and the

incorporation rate returned to control levels 12 h after the introduction of the fungal elicitor.

A comparison of changes in PAL activity (section 3.4) and changes in "PAL" protein synthesis during elicitation revealed that the induction of PAL activity was preceded by the induction of "PAL" protein synthesis. The induction of PAL activity followed the pattern of the accumulation of "PAL" protein and therefore, it was concluded that the observed induction of PAL activity was a consequence of the induction of *de novo* synthesis of the enzyme.

Fungal elicitation induced extractable PAL activity in *Capsicum frutescens* cell cultures via the induction of *de novo* synthesis of the enzyme. Studies at transcriptional level in the next section will show whether the observed changes at translational level are a consequence of changes in PAL gene expression caused by fungal elicitation.

3.6 Investigation of PAL mRNA levels during elicitation of *Capsicum frutescens* suspension cultures

The results presented in section 3.5 showed that ³PAL protein synthesis was induced during elicitation of *Capsicum frutescens* cell cultures. A comparison of ³PAL protein synthesis and extractable PAL activity during elicitation suggested that the observed induction of PAL activity was a consequence of the induction of ³PAL protein synthesis. In this section *Capsicum* PAL mRNA will be identified in order to study the induction of PAL at the transcriptional level. PAL mRNA steady state levels will be followed during elicitation of *Capsicum* cell cultures to discover whether the observed induction of ³PAL protein synthesis is correlated to increased levels of PAL mRNA.

3.6.1 RNA extraction and RNA quality

Total RNA was extracted from frozen *Capsicum* cells as described in section 2.15 and nucleic acids were quantified by UV-spectroscopy. The described extraction procedure routinely yielded *ca.* 80 µg RNA per g FW. Wavelength scans (Fig. 2.15.1) of isolated total RNA indicated the high purity of the nucleic acid preparations. The ratios OD₂₆₀/OD₂₃₀ and OD₂₆₀/OD₂₈₀ were approximately 2.0 for all extractions. Contamination with protein, phenol, or carbohydrate would have altered the ratios considerably (see legend of Fig. 2.15.1). The intactness of the isolated RNA was confirmed by agarose gel electrophoresis (section 2.18.2). The bulk amount of total RNA consists of ribosomal RNA. Therefore, ethidium bromide stained agarose gels of undegraded total RNA predominantly show the two rRNA bands (Fig. 3.6.1, lane A). Ribonuclease activity in RNA preparations would result in a smear of RNA fragments (Fig. 3.6.1, lane D).

Translational activity of RNA extracted from *Capsicum* cells was tested using a rabbit reticulocyte lysate cell free translation system (section 2.17).

Immunoprecipitation of *in vitro* translation products was not an objective because of the inconclusiveness of the method (section 3.5.1.1), however, *in vitro* translational activity of isolated RNA is an indication of the intactness of the RNA.

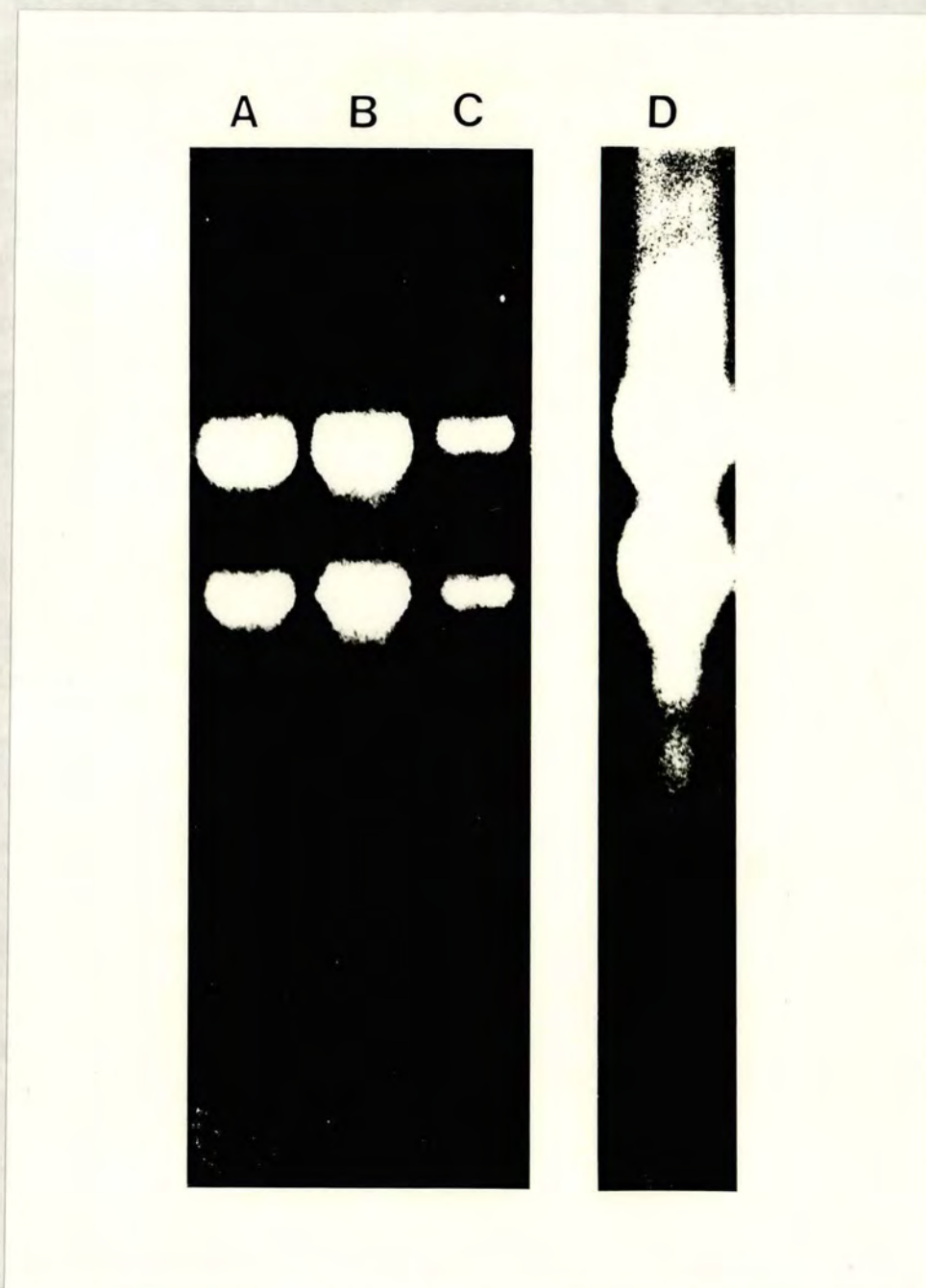


Figure 3.6.1 Denaturing agarose gel electrophoresis of *Capsicum frutescens* RNA: (A) total RNA, (B) poly(A)⁻RNA, (C) poly(A)⁺RNA after one passage through oligo(dT)-cellulose, (D) total RNA degraded by ribonuclease activity.

Total RNA was separated into poly(A)⁺RNA and poly(A)⁻RNA by affinity chromatography on oligo(dT)-cellulose (section 2.16) exploiting the fact that almost all mRNA is polyadenylated at the 3' end (Schleif and Wensink, 1981). Elution profiles from the column chromatography are shown in Fig. 3.6.2. The poly(A) tail of poly(A)⁺RNA annealed to the oligo(dT) residues in the presence of 0.5 M LiCl, while poly(A)⁻RNA did not bind and was washed off the column. A-T duplexes dissociated in the absence of LiCl and the poly(A)⁺ fraction was eluted from the column. RNA eluted from oligo(dT)-cellulose still contained considerable amounts of rRNA. Fig. 3.6.1 shows a comparison of electrophoretically size separated total RNA (lane A), poly(A)⁻RNA (lane B), and poly(A)⁺RNA after one passage of affinity chromatography (lane C). Ribosomal RNA bands are clearly visible in the poly(A)⁺ fraction, although strongly reduced in quantity. It is common practice to subject poly(A)⁺RNA to a second cycle of affinity chromatography (Sambrook *et al.*, 1989). RNA eluted from the second oligo(dT)-cellulose column was not analysed by agarose gel electrophoresis because of the small amount obtained.

In vitro translation time course experiments (section 2.17) using TMV mRNA were performed to study the rate of [³⁵S]methionine incorporation into translation products. Aliquots (1 µl) of the translation mixtures were sampled at various time intervals and the synthesised polypeptides were analysed by LSC (section 2.7.2) for [³⁵S]methionine incorporation. The results are shown in Fig. 3.6.3 where incorporation of radiolabel into protein is plotted against time. Incorporation rose sharply at the start of the reaction and reached a plateau after *ca.* 1 h reaction time. Therefore, 1 h incubation time was considered sufficient to obtain a constant rate of radiolabel incorporation. Incorporation rates were negligible in control samples without exogenous RNA (-TMV mRNA).

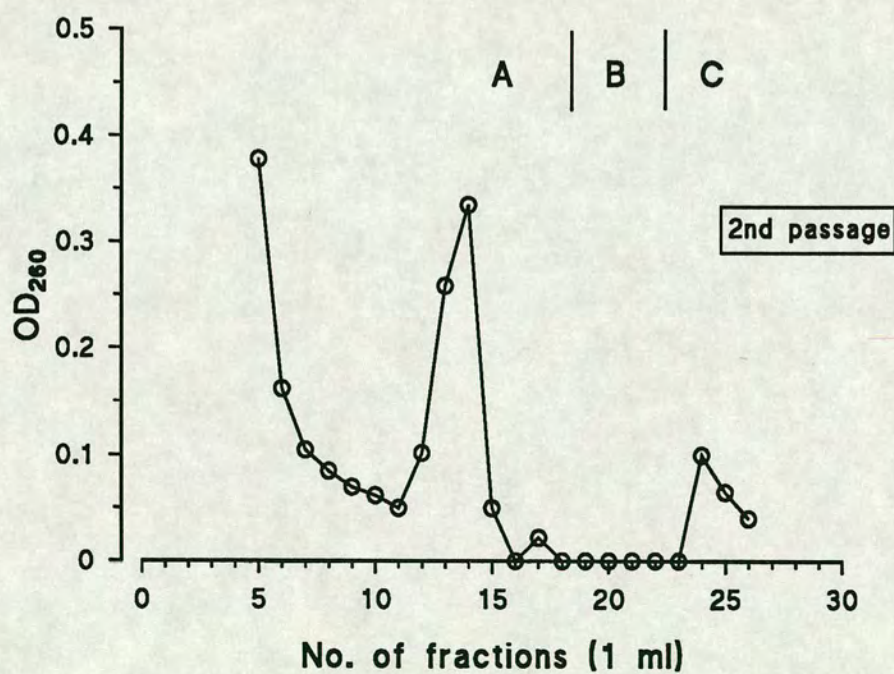
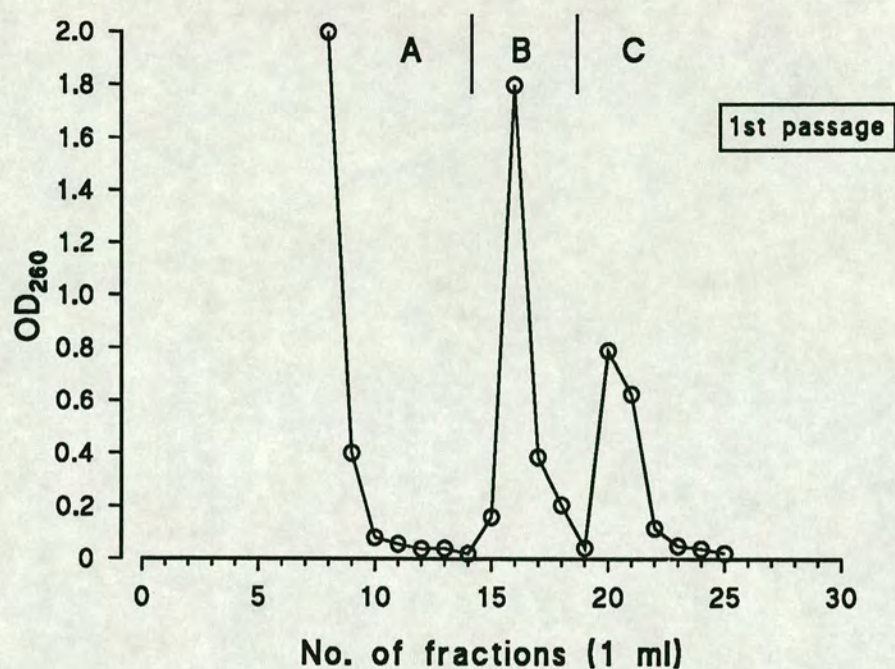


Figure 3.6.2 Elution profiles of oligo(dT)-cellulose columns. (A)= Binding, (B)= Washing, (C)= Elution. Constituents of the buffers are listed in section 2.16. Fractions 20-23 of the 1st passage were combined and reapplied to a regenerated column. Fraction 24 of the 2nd passage was used for *in vitro* translation experiments.

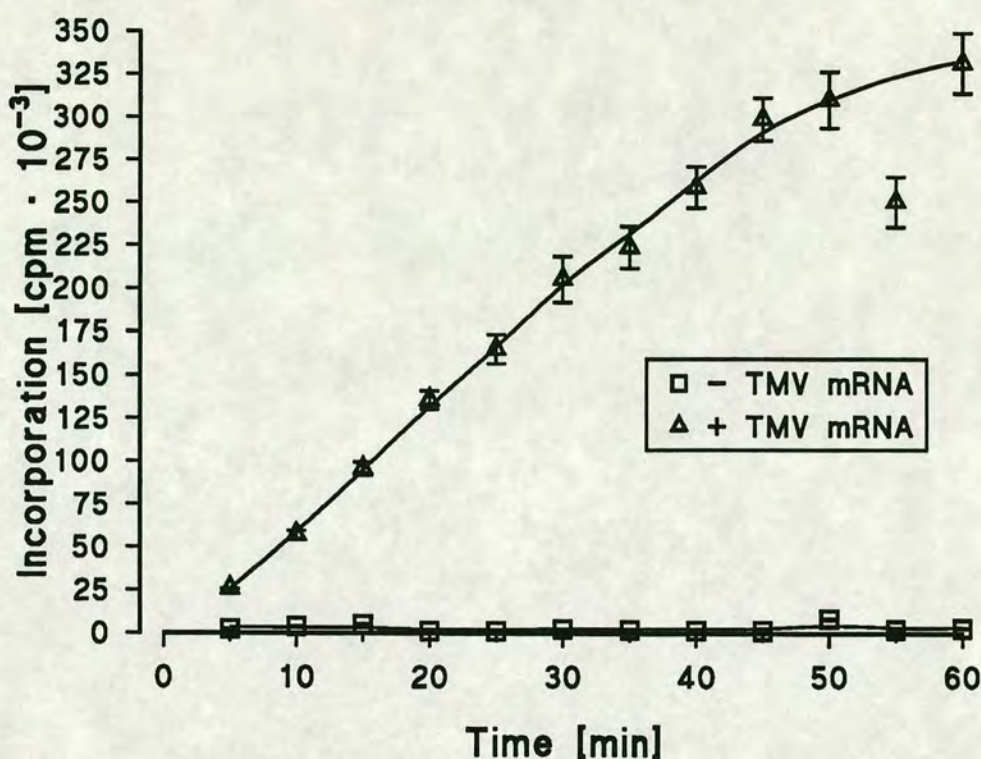


Figure 3.6.3 The time course of [^{35}S]methionine incorporation into TCA precipitable translation products in a rabbit reticulocyte lysate cell free translation system was followed using TMV mRNA. Incorporation rates in the absence of exogenous RNA were negligible. Each datapoint represents the arithmetic mean of three replicates \pm SE

In vitro translations in the rabbit reticulocyte lysate cell free translation system were performed using *Capsicum* poly(A)⁺RNA and *Capsicum* poly(A)⁻RNA. TMV mRNA or water replaced *Capsicum* RNA for positive and negative controls, respectively. The reaction was stopped after 1 h and the samples were analysed by SDS-PAGE (section 2.8). Incorporation of [^{35}S]methionine into TCA precipitable polypeptides was determined by LSC (section 2.7.2). Incorporation rates were 50-60% for TMV mRNA, 5% for *Capsicum* poly(A)⁺RNA, 1% for *Capsicum* poly(A)⁻RNA, and 0.5% for water. Radiolabelled translation products, size separated by SDS-PAGE, were detected by fluorography (section 2.11.2). The band visible in the minus mRNA track (Fig. 3.6.4, lane A) is a common phenomenon and is probably due to labelling of cold proteins or other macromolecules by an mRNA independent process

(manufacturer's manual). Note, the same band (size 40-50 kD) is present in all samples.

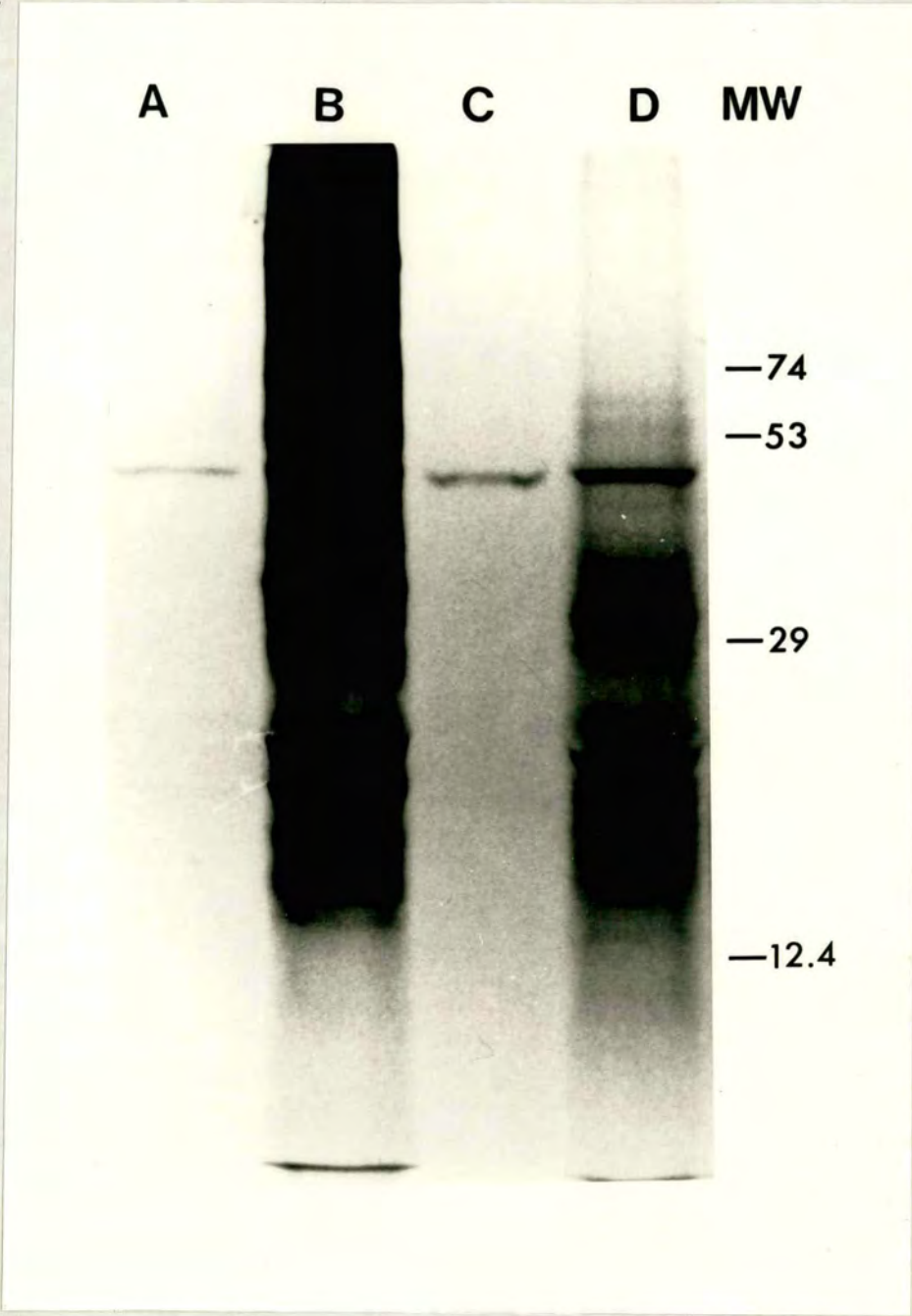


Figure 3.6.4 Fluorograph of [³⁵S]methionine labelled *in vitro* translation products, size separated by SDS-PAGE. (A) Negative control (water instead of exogenous RNA), (B) positive control (TMV mRNA), (C) *Capsicum* poly(A)-RNA, (D) *Capsicum* poly(A)+RNA.

Apart from this band no incorporation was detected by fluorography for *Capsicum* poly(A)-RNA (Fig. 3.6.4, lane C). The incorporation rate of 1%, as estimated by LSC of TCA precipitable material, seemed mainly due to the mRNA independent labelling process already mentioned. *Capsicum* poly(A)+RNA coded for a range of polypeptides (Fig. 3.6.4, lane D) thus, affirming the predicted *in vitro* translation activity of the RNA preparations. These findings confirmed the presumed intactness and quality of the extracted RNA indicated by agarose gel electrophoresis analysis and UV-spectroscopy. Higher rates of incorporation and more synthesis of high molecular weight polypeptides are likely after optimisation of the cell free translation system for *Capsicum* mRNA, especially incubation time, ion and RNA concentrations in the reaction mixture.

3.6.2 Identification and characterisation of *Capsicum* PAL mRNA

Capsicum PAL mRNA was identified using a parsley PAL cDNA probe. Parsley PAL cDNA was obtained as a clone (pcPAL 35B) in *Escherichia coli* strain MC 1061 (section 2.3.3). The cDNA had been cloned into the *EcoRI* site of the commercially available vector pBS M13+ which also carries the genes for ampicillin resistance and β -galactosidase. The position of the cDNA insert in the multiple cloning site of the vector is indicated in Fig. 3.6.5.

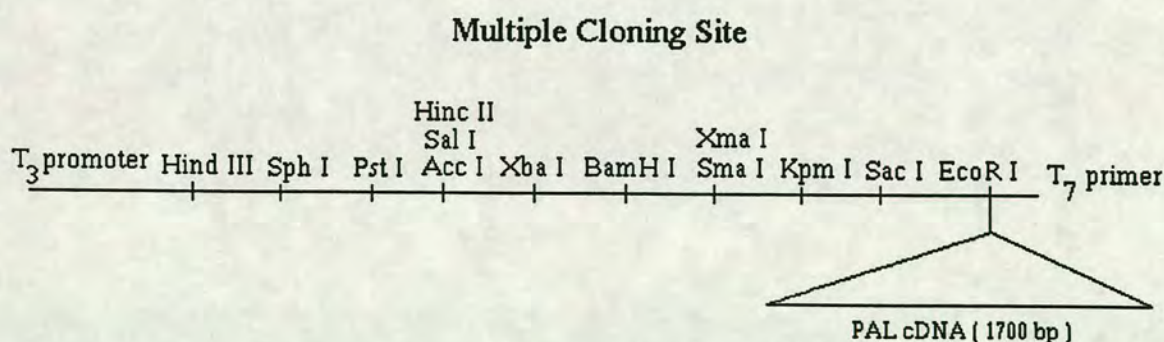


Figure 3.6.5 Parsley PAL cDNA (1700 bp) is inserted into the *EcoRI* restriction site of the plasmid's (pcPAL 35B) multiple cloning site. Distances are not drawn to scale.

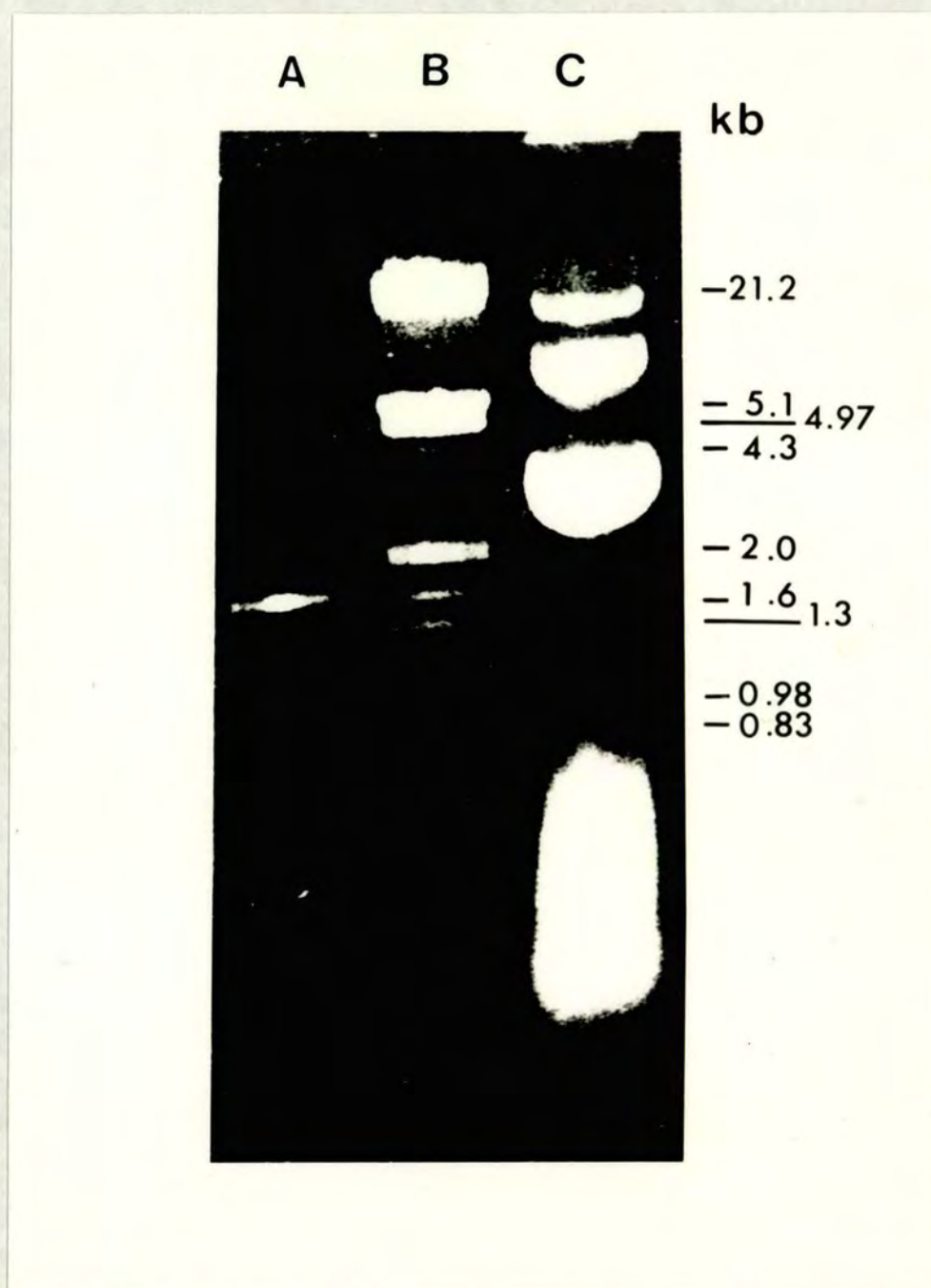


Figure 3.6.6 Non-denaturing agarose gel electrophoresis of DNA. (A) Parsley PAL cDNA insert, (B) λ -DNA restriction fragments produced by *Hind*III + *Eco*RI digestion (Sigma, U.K.), (C) plasmid preparation showing (from top to bottom) relaxed plasmid, coiled plasmid, supercoiled plasmid, and RNA contamination.

E. coli cultures were maintained on medium containing ampicillin as described in section 2.3.3 and plasmids were isolated from overnight cultures using the alkaline ex-

traction method (section 2.20.1). The PAL cDNA insert was excised and separated from the plasmid as described in section 2.20.2. The molecular size of the linearised PAL cDNA insert was estimated by agarose gel electrophoresis (Fig. 3.6.6). Molecular weight markers were DNA restriction fragments (*Hind*III + *Eco*RI digest) of bacteriophage λ (Sigma, U.K.). The size of parsley PAL cDNA was 1700 bp as estimated by interpolation from its distance migrated in 0.7% agarose gels compared to the distance migrated by the λ -DNA restriction fragments run on the same gel (Fig. 3.6.7). The full length of the parsley PAL gene is reported to be 2400 bp (Lois *et al.*, 1989).

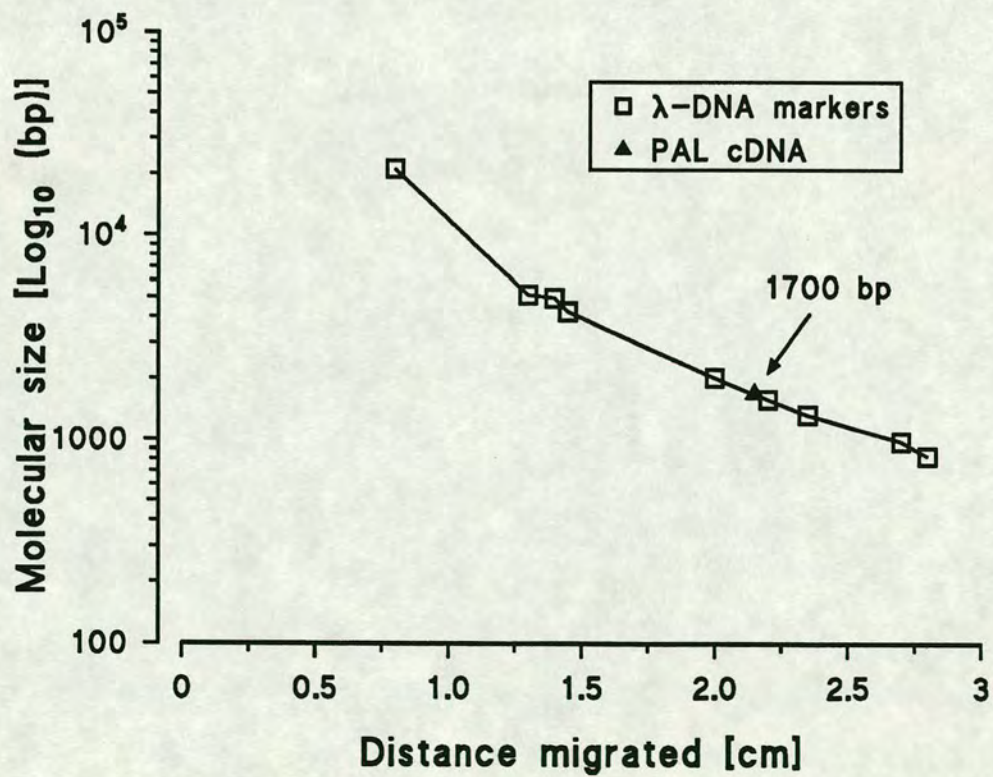


Figure 3.6.7 The molecular size of the parsley PAL cDNA insert was estimated by interpolation from its distance migrated in 0.7% agarose gels compared to the distance migrated by λ -DNA molecular size markers.

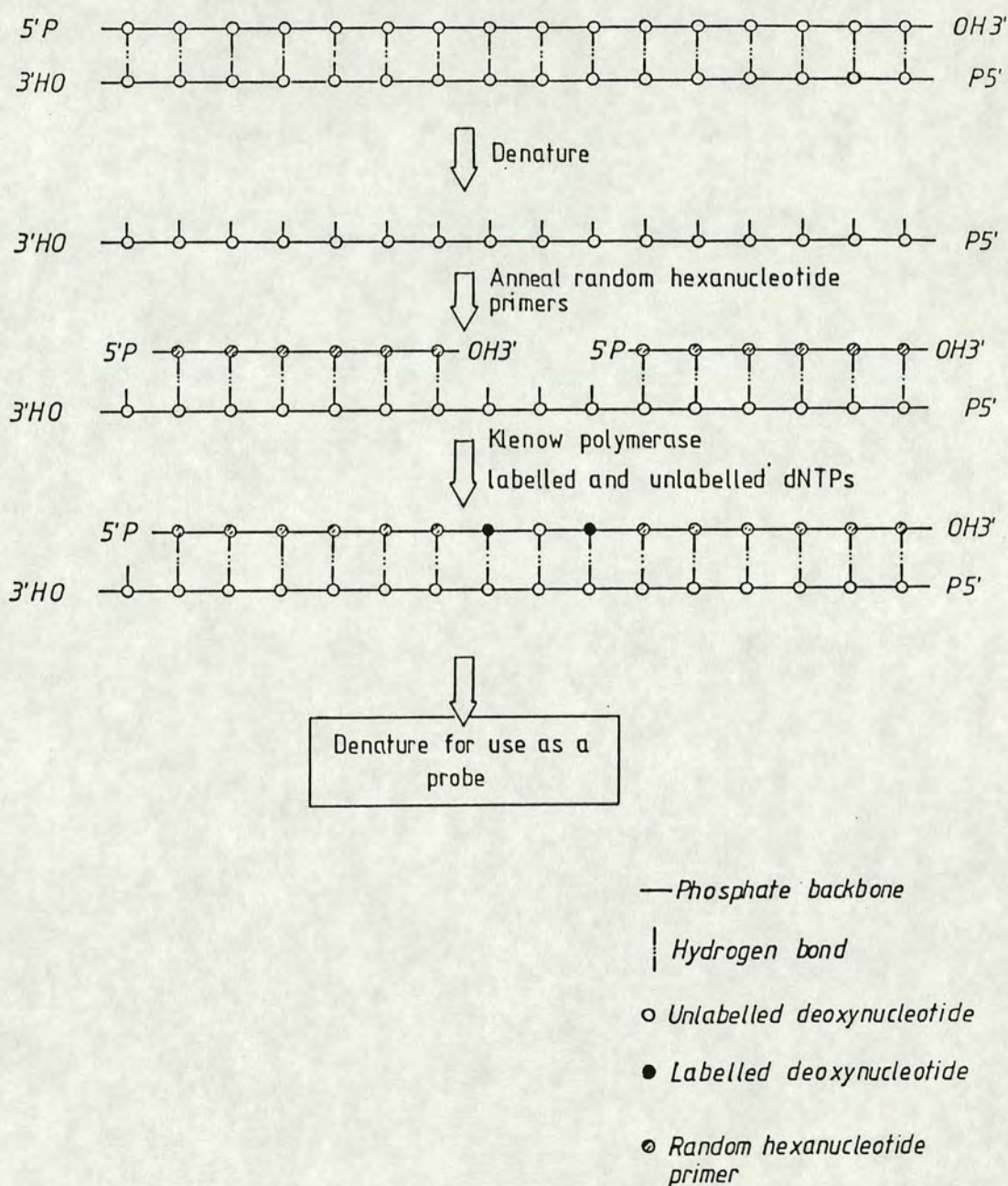


Figure 3.6.8 The random prime labelling reaction, like all primer extension methods, utilises the ability of DNA polymerase to synthesise a new DNA strand complementary to a template strand, starting from a free 3'-hydroxyl. The latter is provided by a short oligonucleotide primer annealed to the template. A mixture of primers of random sequence is used in order to produce a uniformly labelled DNA copy of any sequence. It is essential to use a polymerase lacking a 5'-3' exonuclease activity like the Klenow fragment of *E. coli* DNA polymerase I, otherwise degradation of the primer would occur (Feinberg and Vogelstein, 1983;1984).

PAL cDNA was radioactively labelled with α -[^{32}P]CTP using the random prime labelling reaction as described in section 2.20.3. The principle of the reaction is outlined in Fig. 3.6.8. Labelled PAL cDNA was used as a probe to identify PAL mRNA by Northern Blot analysis.

PAL mRNA synthesis is known to be induced following dilution of plant cell cultures (for review see Jones, 1984). This feature was used to identify *Capsicum* PAL mRNA using the parsley PAL cDNA probe. Total RNA was extracted from *Capsicum* cells before and 2 h after dilution of the cultures. *Capsicum* RNA samples and *E. coli* RNA was run on denaturing agarose gels (section 2.18.2) and blotted onto nylon membrane (section 2.19.1).

Fig. 3.6.9 shows the ethidium bromide stained agarose gel. *E. coli* rRNA bands served as molecular weight markers for the estimation of the size of *Capsicum* rRNA. *E. coli* rRNA is known to be 2.9 kb and 1.5 kb of length for 23 S rRNA and 16 S rRNA, respectively (Lewin, 1987). *Capsicum* rRNA was estimated to be 3.2 kb for 25 S rRNA and 1.8 kb for 18 S rRNA.

PAL mRNA bound to the nylon membrane was hybridised with labelled cDNA (section 2.19.2) and radioactivity on the Northern Blot was located by fluorography (section 2.11.2). The results are shown in Fig. 3.6.10.

No hybridisation was detected with *E. coli* RNA (Fig. 3.6.10, lane C) which served as a negative control. Undiluted *Capsicum* cultures (Fig. 3.6.10, lane B) exhibited a low level of PAL mRNA. Following 2 h dilution of the cultures, PAL mRNA levels increased considerably as estimated by the amount of hybridisation with the labelled PAL cDNA probe (Fig. 3.6.10, lane A).

The peak areas of densitometer scans of the fluorographs were calculated as 0.48 cm² for undiluted cultures and 4.73 cm² for diluted cultures. This indicated that PAL mRNA levels in diluted cultures were *ca.* 10 times the levels detected in control cultures.

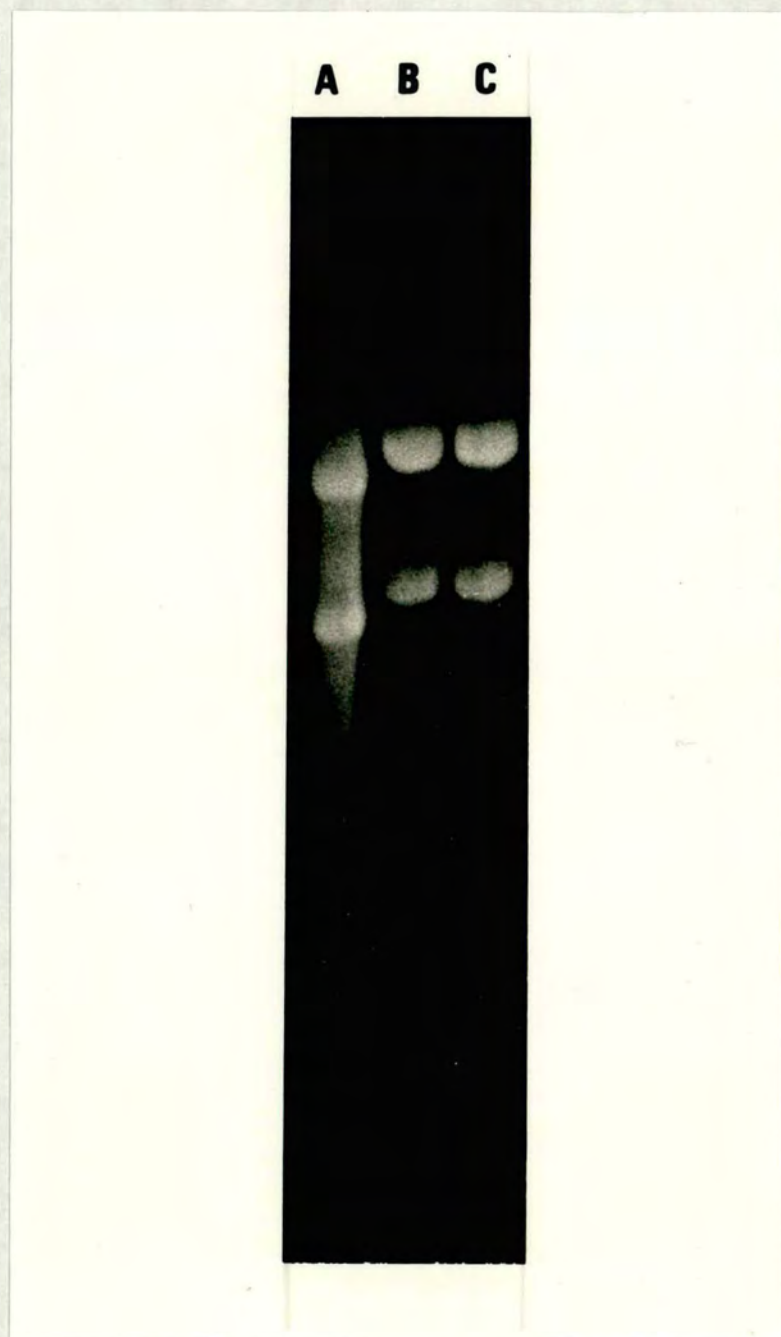


Figure 3.6.9 Denaturing agarose gel electrophoresis of total RNA extracted from (A) *E. coli*, (B) undiluted *Capsicum* cultures, and (C) diluted *Capsicum* cultures. 10 μ g RNA were loaded on each lane.

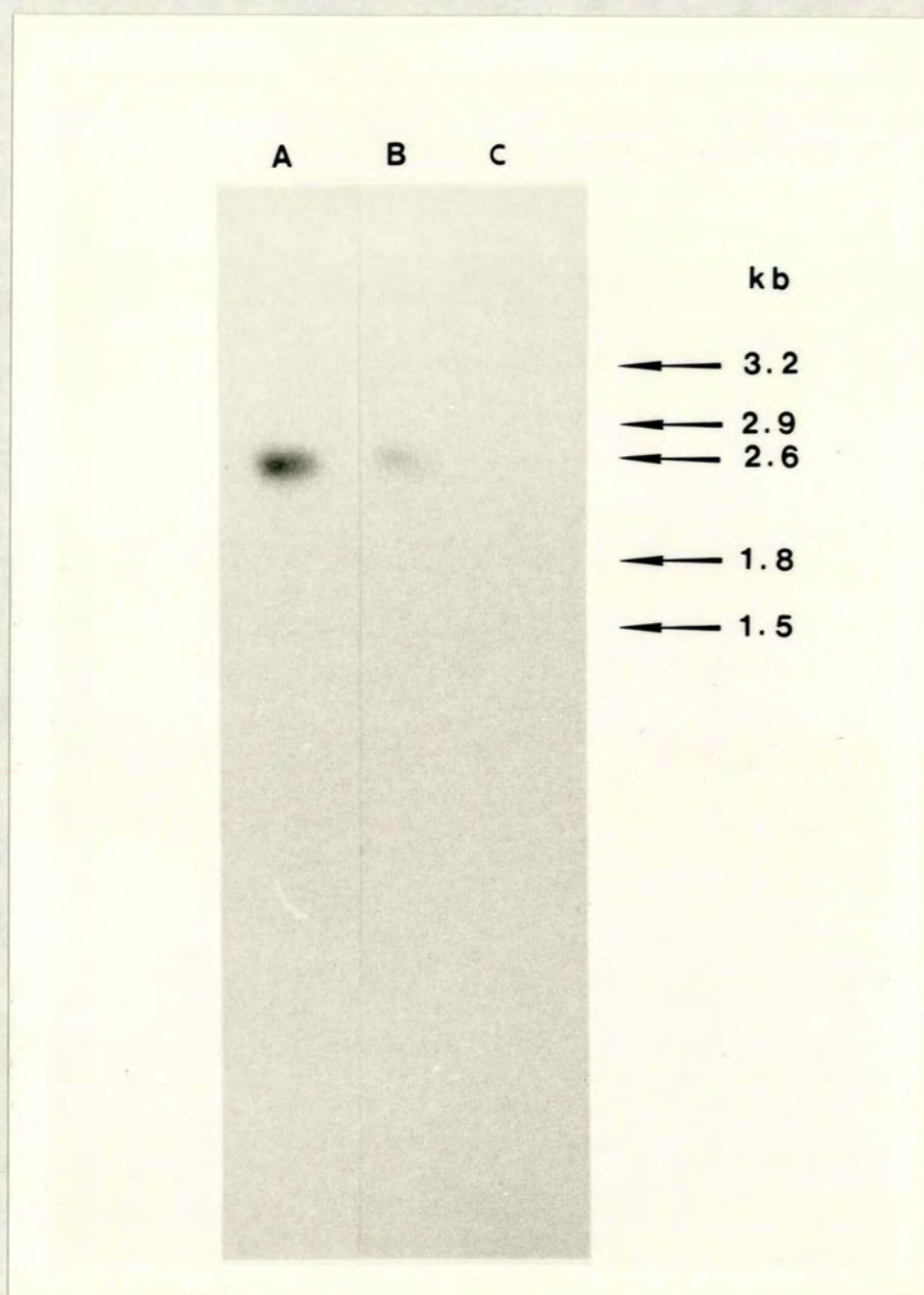


Figure 3.6.10 *Capsicum* PAL mRNA hybridised to labelled parsley PAL cDNA was detected by fluorography. (A) RNA extracted 2 h after dilution of *Capsicum* cultures, (B) RNA extracted from control cultures, (C) *E. coli* RNA. Positions and molecular sizes of *Capsicum* and *E. coli* rRNA bands and PAL mRNA are indicated.

No unspecific hybridisation of the cDNA probe was detected by fluorography of the Northern Blot. However, scanning of the membrane using a flow detector showed that some activity was associated to rRNA, especially to 18 S rRNA (Fig. 3.6.11). This is considered a common phenomenon and does not reflect a lack of specificity of the cDNA probe (S.M. Smith, personal communication). The amount of radioactivity associated with rRNA bands was too small to be detected by fluorography during the overnight exposure and was consequently considered negligible for analysis of the Northern Blots.

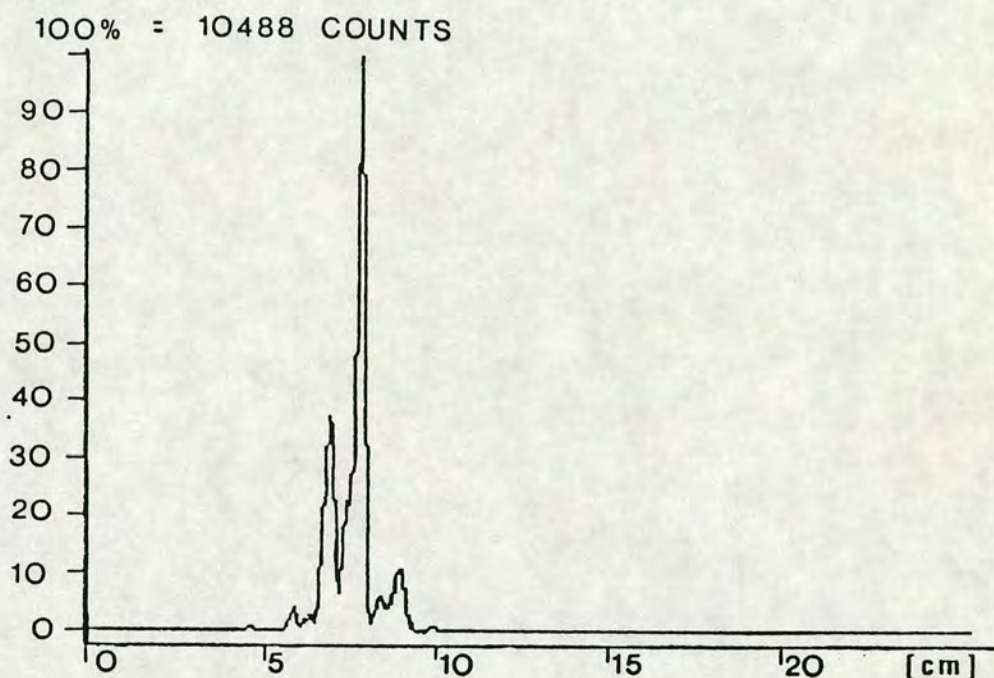


Figure 3.6.11 Scanning of lane A of Fig. 3.6.10 using a flow detector showed that radioactivity was associated to PAL mRNA (centre peak) and to both *Capsicum* rRNA bands. The scan operated in reverse direction thus, the first peak indicating 18 S rRNA.

The apparent lack of unspecific hybridisation as detected on the fluorograph in conjunction with the high stringency of the washing procedures suggested a high homology of the parsley PAL cDNA and *Capsicum* PAL mRNA.

The ethidium bromide stained *Capsicum* and *E. coli* rRNA bands were visible under UV-light and their positions and estimated molecular sizes are indicated in Fig.

3.6.10. The size of PAL mRNA (2.6 kb) was estimated by its distance migrated in the agarose gel relative to the distance migrated by the rRNA markers. DNA restriction fragments are not suitable for RNA size determinations because of their different migration properties. Commercially available synthetic RNA molecular size markers (RNA "ladders") were not considered an option due to the cost in relation to the small improvement in accuracy.

The above results show;

- 1) The suitability of parsley PAL cDNA as a probe for *Capsicum* PAL mRNA.
- 2) The identification and size estimation (2.6 kb) of *Capsicum* PAL mRNA using Northern Blotting.
- 3) The increase in PAL mRNA following dilution of *Capsicum* suspension cultures.

3.6.3 PAL mRNA levels during elicitation

PAL mRNA levels during elicitation of *Capsicum frutescens* cell suspensions were studied using Northern Blot analysis. *Capsicum* cultures were elicited for 20 min, 40 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h using a fungal elicitor preparation (section 2.4). Total RNA was extracted as described in section 2.15 and aliquots (10 µg) were subjected to denaturing agarose gel electrophoresis (section 2.18.2). RNA was transferred to nylon membrane (section 2.19.1) and hybridised with radioactively labelled PAL cDNA (section 2.19.2). Hybridisation of labelled cDNA probe and PAL mRNA was detected by fluorography (section 2.11.2). The results are presented in Fig. 3.6.12 and Fig. 3.6.13.

In control cultures, without elicitor treatment, PAL mRNA was detectable at a low level (Fig. 3.6.12 and Table 3.6.1). The amount of detectable PAL mRNA approximately doubled during the first 20 min of elicitation and increased further to

reach its maximum *ca.* 4 h after the introduction of the fungal elicitor. The amount of PAL mRNA detected 4 h after the onset of elicitation was *ca.* 6-8 times the amount detectable in control cultures as estimated by densitometer scans (Table 3.6.1). Following the maximum, detected after 4 h, PAL mRNA levels in *Capsicum* cultures decreased despite the continuous presence of the fungal elicitor. Between 4 h and 8 h after elicitation the amount of PAL mRNA decreased by *ca.* 50%.

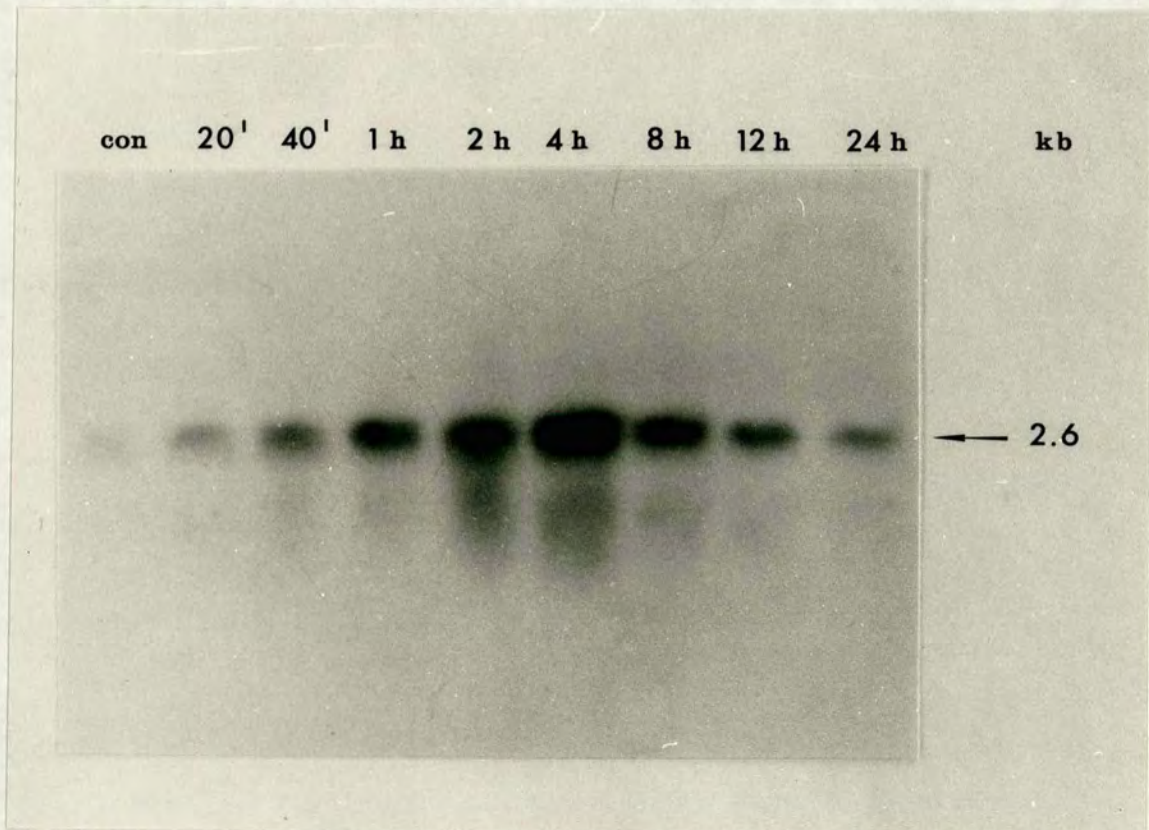


Figure 3.6.12 Northern Blot analysis of elicitation time course experiment. Hybridisation of labelled cDNA probe with PAL mRNA was detected by fluorography. Elicitation times and the molecular size of PAL mRNA are indicated. Control cultures (con) did not receive elicitor treatment.

At the end of the experiment (24 h) PAL mRNA levels had returned to the amounts estimated for $t_{20\text{min}}$ (Table 3.6.1). Thus, indicating the transiency of the observed increase in steady state levels of PAL mRNA following elicitation of *Capsicum* cell cultures.

Table 3.6.1 Fluorographs of Northern Blots (Fig. 3.6.12) were quantified by densitometer scans. Absorption (OD_{540}) of the PAL mRNA band on the fluorographs is expressed as peak area (relative units).

time	PAL mRNA [rel. units]	% of maximum value
0 min	4807	14.7
20 min	8519	26.1
40 min	11186	34.3
1 h	17016	52.1
2 h	27518	84.3
4 h	32650	100.0
8 h	17356	53.2
12 h	12527	38.4
24 h	9047	27.7

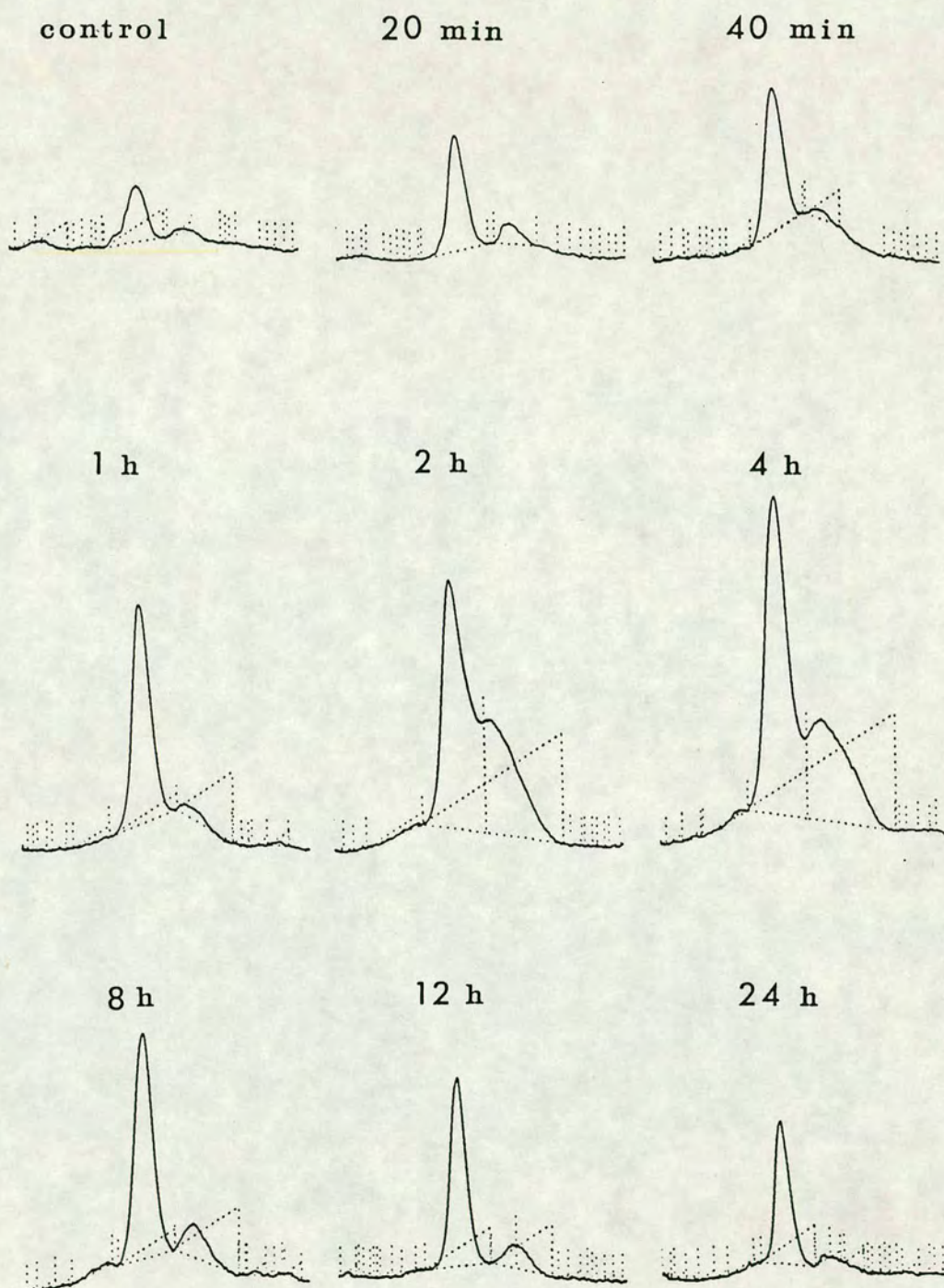


Figure 3.6.13 The intensity of the PAL mRNA band visible on the fluorograph (Fig. 3.6.12) was quantified by densitometer scans. The absorption at 540 nm wavelength gives an indication of the blackening of the X-ray film. Elicitation times are indicated above the scans.

3.6.4 Effects of fungal elicitation on PAL mRNA levels, "PAL" protein levels, and PAL activity

PAL mRNA levels during elicitation of *Capsicum frutescens* cell cultures were followed using Northern Blot analysis. Increased levels of PAL mRNA were detected 20 min after the introduction of the fungal elicitor. The highest PAL mRNA levels were measured after 4 h and they decreased thereafter despite the continuous presence of the fungal elicitor thus, indicating the transiency of the effect. PAL mRNA levels during elicitation are compared with PAL activity (section 3.4) and "PAL" protein levels (section 3.5) in Fig. 3.6.14.

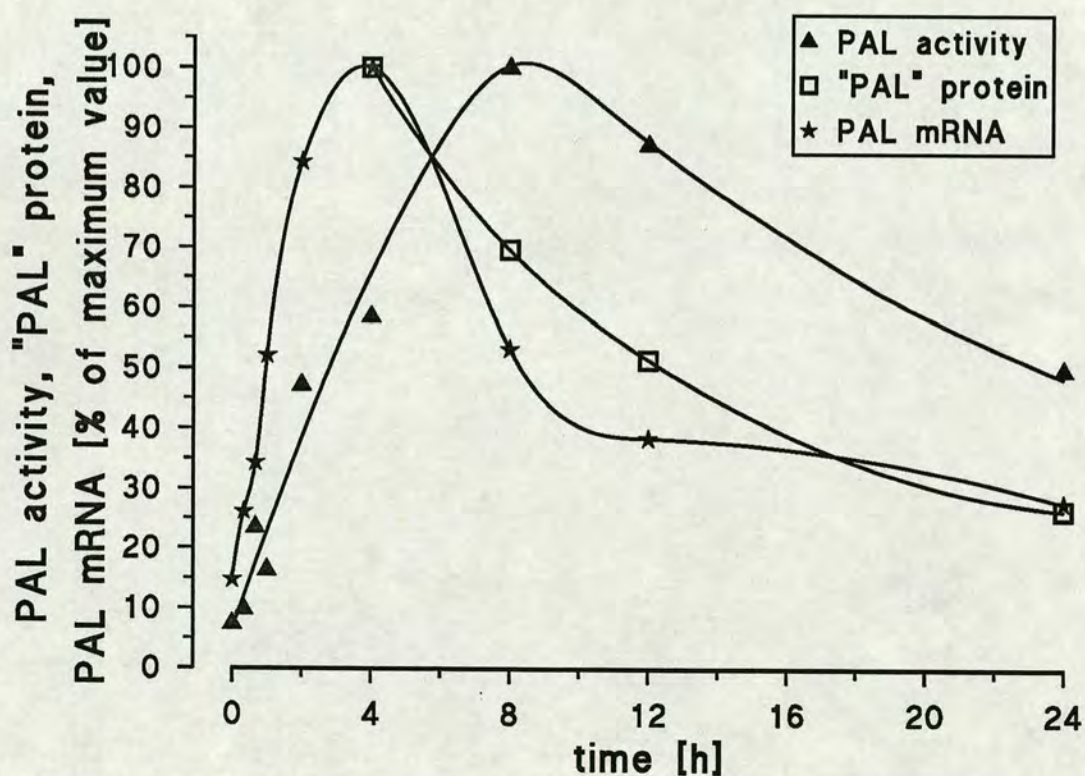


Figure 3.6.14 PAL activity and levels of "PAL" protein and PAL mRNA during elicitation of *Capsicum frutescens* cell cultures are expressed relative to the highest amount observed.

The observed increase in PAL mRNA levels during the first 4 h of elicitation is steeper than the increase observed for "PAL" protein levels during the same time period. PAL mRNA levels 2 h after the onset of elicitation were an estimated 84.3% of the maximum level measured after 4 h and dropped to 53.2% after 8 h (Table 3.6.1). The skewness of the resulting curve (Fig. 3.6.14) suggests that the "true" maximum lies between 2 h and 4 h after elicitation. Therefore, increased PAL mRNA levels precede the observed increase in "PAL" protein which, in turn, is succeeded by increased PAL activity.

3.6.5 Summary

In section 3.6 PAL mRNA steady state levels were followed during elicitation of *Capsicum frutescens* cell cultures. Therefore, preliminary studies regarding the quality of the RNA preparations were necessary. Agarose gel electrophoresis and *in vitro* translation experiments confirmed that the extracted RNA was intact. *In vitro* translation activity was found to be solely associated with poly(A)⁺RNA. A parsley PAL cDNA of 1700 bp length enabled the identification of *Capsicum* PAL mRNA using Northern Blot analysis. The cDNA probe was shown to be specific for PAL mRNA. The molecular size of PAL mRNA was estimated to be 2.6 kb.

Dilution of *Capsicum* cultures was found to increase PAL mRNA steady state levels. Two hours after a 1:1 dilution, PAL mRNA levels were found to be increased *ca.* 10 times compared to the controls.

Elicitation of *Capsicum* cultures using a fungal elicitor preparation transiently increased PAL mRNA levels. Increased levels were detected 20 min after the introduction of the fungal elicitor and the highest levels were measured after 4 h.

A comparison of PAL activity, "PAL" protein, and PAL mRNA levels during elicitation showed that the fungal elicitor preparation transiently increased PAL mRNA levels resulting in *de novo* synthesis of "PAL" protein and thus, increasing PAL activity.

PAL is known to play an important role in the phenylpropanoid metabolism of plant cells. Therefore, in the next section the effects of elicitation and increased PAL activity on the synthesis of soluble phenolic compounds will be studied.

3.7 Incorporation of [^{14}C]phenylalanine into free phenolic compounds during elicitation of *Capsicum frutescens* cell cultures

PAL is known to play a key role in the phenylpropanoid metabolism of plants (for a review see Hahlbrock and Scheel, 1989). Therefore, the observed changes in PAL activity following elicitation of *Capsicum* cell cultures are likely to affect the biosynthesis of phenolic compounds. Capsaicin biosynthesis, under investigation in this laboratory, is closely linked to general phenylpropanoid metabolism because the aromatic moiety of capsaicin is derived from phenylalanine (Fig. 1.4.2) and the major part of this branch of the pathway (from phenylalanine to ferulic acid) forms the core of reactions in the phenylpropanoid metabolism of plant cells.

The experiments described in this section were designed to follow changes in phenylpropanoid metabolism during elicitation of *Capsicum frutescens* cell cultures. The aromatic branch of the pathway leading to capsaicin was chosen as a model system with attention focussed on the pattern of free phenolic compounds as they are the supposed intermediates in the synthesis of the aromatic moiety of capsaicin. Changes in the flux of metabolites down this branch of the capsaicin pathway during elicitation were followed using L-[U- ^{14}C]phenylalanine. Stationary-phase *Capsicum* cultures (21 d after sub-culture, see section 3.2.3.6) were used for the labelling experiments, because (1) the down-turn of growth often creates favourable conditions for secondary metabolism (Yeoman *et al.*, 1980; Lindsey and Yeoman, 1985) and (2) PAL activity responded strongest to elicitation at this stage of the growth cycle (section 3.3.3). The first set of experiments (section 3.7.1) studies the incorporation of [^{14}C]phenylalanine into free phenolics during the first 24 h of the elicitor treatment. In section 3.7.2 long term (72 h) effects of fungal elicitation on the synthesis of phenolic intermediates were investigated.

3.7.1 Short term effects of elicitation on phenylpropanoid metabolism

The expression "short term" denotes the time interval following the introduction of the fungal elicitor during which PAL activity is transiently increased, i.e. 0-24 h. Fungal elicitor preparation and 5 μCi [^{14}C]phenylalanine were added simultaneously to the experimental flasks. Elicitation (and labelling) periods were 4 h, 8 h, 12 h, and 24 h, respectively. Control cultures were incubated with 5 μCi [^{14}C]phenylalanine in the absence of fungal elicitor for 24 h before harvest. Cells and medium were analysed separately for incorporation of radiolabel into free phenolic compounds using TLC and HPLC. The addition of non-radioactive carriers during the extraction procedure (sections 2.12.2, 2.12.3) was omitted for this set of experiments.

Table 3.7.1 Radioactivity in the organic extracts and in the culture medium after extraction was determined using LSC. The elicitation periods indicated are identical with the labelling periods except for controls (0 h) which were labelled for 24 h in the absence of fungal elicitor.

	Radioactivity [$\text{Bq} \cdot 10^{-3}$]				
	0 h	4 h	8 h	12 h	24 h
medium extract	7.971	0.969	4.590	1.686	0.958
cell extract	13.873	47.792	28.328	10.975	10.695
residue in medium	10.525	21.723	20.550	52.961	13.470
total (% total activity added)	32.369 (16%)	70.484 (38%)	53.468 (30%)	65.622 (35%)	25.123 (14%)

3.7.1.1 Extracts from culture medium

Phenolic compounds were extracted from culture medium and cell washes as described in section 2.12.2, except that a mixture of diethyl ether:ethylacetate (1:1) was used instead of chloroform. Radioactivity in the organic extracts and residual activity in the medium after extraction was determined using LSC (Table 3.7.1). The total amount of radioactivity detected in these fractions varied corresponding to the length of the labelling period. Labelling periods of 24 h yielded 14-16% of the applied radio-label in the organic extracts and the medium after extraction. Shorter labelling periods (4 h, 8 h, 12 h) yielded higher amounts (30-38%) in these fractions.

3.7.1.1.1 Analysis of phenolic compounds by TLC

Samples of the phenolic compounds extracted from the culture medium were analysed using 2D-TLC. The TLC plates were loaded with 30 μl sample and 20 μl non-radioactive standards (400 $\mu\text{g}\cdot\text{ml}^{-1}$ capsaicin and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ each of caffeate, cinnamate, coumarate, ferulate, vanillin, and vanillylamine). The phenolic compounds were eluted using benzene:acetic acid (9:2, 1st dimension) and *iso*-propanol:butanol:ammonia:water (6:2:1:1, 2nd dimension) as described in section 2.12.4. Upon drying, the plates were subjected to direct autoradiography (section 2.11.1) using an exposure period of 21 d. Following autoradiography, the phenolic compounds were visualised as described in section 2.12.4. A second set of TLC plates was loaded and eluted as above. The phenolic compounds of this set were visualised by exposing the plates to iodine fumes in a sealed glass tank. Fumes of iodine are a general visualising agent for compounds with double bonds (substitution reaction). The detection method with iodine was chosen because, it does not interfere with radioactivity measurements using LSC (Sukrasno, 1991). Iodine stained spots of phenolic standards were scraped off the TLC plates and radioactivity was measured using LSC (section 2.7.2).

Fig. 3.7.1 is a series of autoradiographs showing the 2D-TLC separation of [^{14}C]phenylalanine and various labelled phenolic compounds. Labels A-H indicate radioactive compounds that comigrated with non-radioactive phenolic standards (see figure legend). Radioactive spots without a label denote unidentified compounds.

[^{14}C]Phenylalanine (label A, Fig. 3.7.1) was conditionally identified by its characteristic shape and its position relative to the origin (label O) and to the other phenolic standards. The mixture of standards applied did not contain phenylalanine.

The radioactive compound that comigrated with vanillylamine (label F, Fig. 3.7.1) was not identical with [^{14}C]vanillylamine for the following reasons. (1) The compound comigrated with vanillylamine standards in all extracts except one (4 h). Like vanillylamine, the unidentified compound was not eluted by the first (non-polar) solvent system. In the second solvent system the compound migrated a little slower than the vanillylamine standard thus, resulting in a final position slightly to the left of vanillylamine. (2) Vanillylamine was not extracted when large amounts were added as non-radioactive carrier during the extraction procedure (section 3.7.2.2). Therefore, it can be concluded that the compound in question is not [^{14}C]vanillylamine. The observed comigration with vanillylamine is considered to be coincidental.

Cinnamic acid, the product of PAL activity, was present in extracts from unelicited (0 h) cultures and in cultures that were elicited for 4 h (label B, Fig. 3.7.1). [^{14}C]Cinnamic acid could neither be detected in cultures elicited for 8 h, when PAL activity was highest (section 3.4.3), nor at later stages during the elicitation time course.

[^{14}C]Coumaric acid (label C, Fig. 3.7.1) was detected in the medium of *Capsicum* cultures elicited for 4-12 h, but was neither present in cultures elicited for 24 h nor in controls.

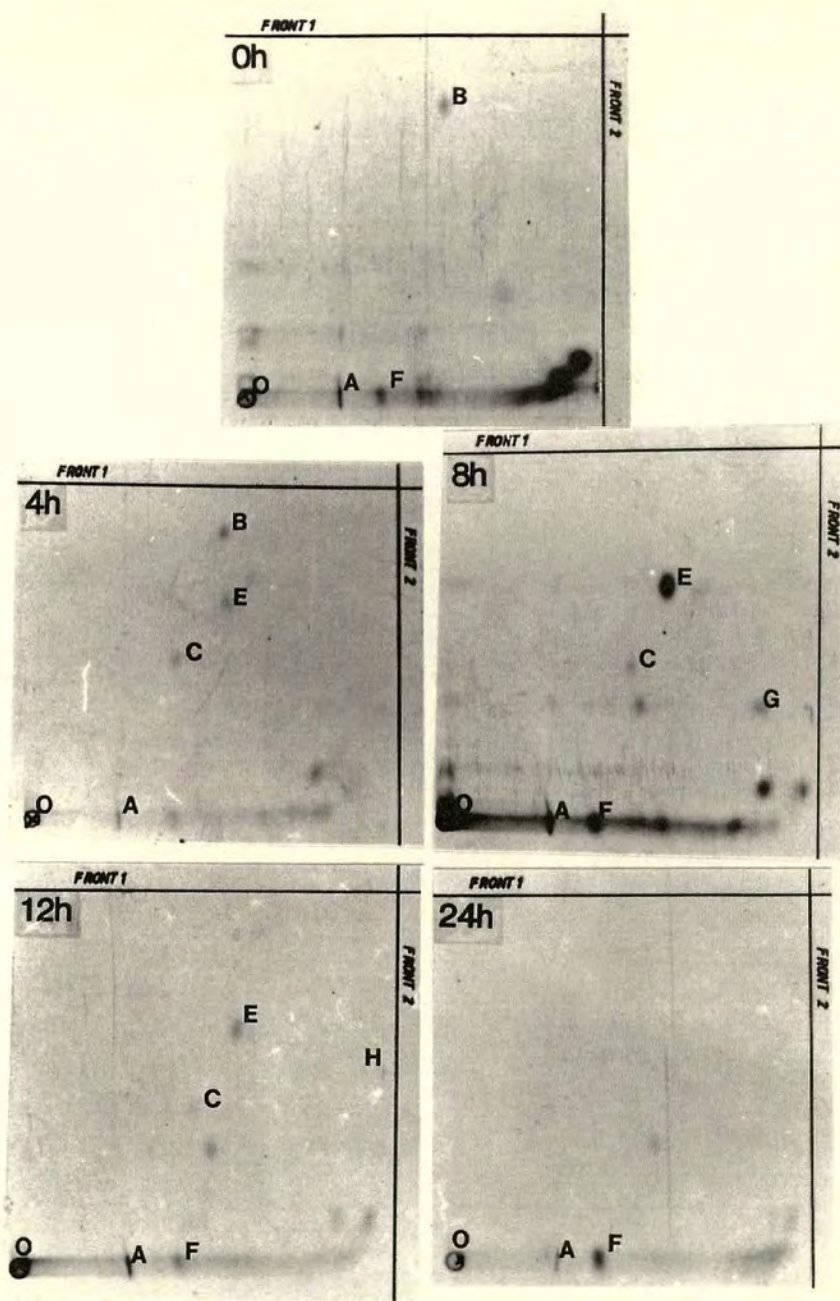


Figure 3.7.1 Phenolic compounds extracted from the culture medium separated by 2D-TLC and analysed for $[^{14}\text{C}]$ incorporation using direct autoradiography. The elicitation times indicated in the top left corner of each autoradiograph were identical with the labelling periods, except for controls (0 h) which were labelled for 24 h in the absence of fungal elicitor. The solvent fronts are marked at the top (benzene:acetic acid, 9:2) and at the right hand side (*iso*-propanol:butanol:ammonia:water, 6:2:1:1) of each autoradiograph. The starting point (origin = O) of the separation is situated in the bottom left corner of each autoradiograph. Radioactive compounds that comigrated with non-radioactive standards are labelled as follows. A = phenylalanine (see text), B = cinnamic acid, C = coumaric acid, D = ferulic acid, E = vanillin, F = vanillylamine, G = vanillyl alcohol, H = capsaicin.

[^{14}C]Vanillin (label E, Fig. 3.7.1) was detected in the medium of cultures that were elicited for 4 h, 8 h, and 12 h. The highest incorporation of radiolabel into vanillin was observed in cultures elicited for 8 h (Fig. 3.7.1). [^{14}C]vanillyl alcohol was detected in the same extract.

A very faint spot for [^{14}C]capsaicin was detected on the autoradiograph of medium extracts from 12 h elicited cultures. A comparison of the radioactivity measured in the capsaicin standards that were scraped off the TLC plates (Table 3.7.2) showed, that there was very little incorporation into capsaicin in any of the cultures. The highest amount of radioactivity incorporated into capsaicin was measured in extracts from 8 h elicited cultures and not in extracts from 12 h elicited cultures. The very faint signal for [^{14}C]capsaicin on the autoradiograph of 12 h elicited cultures could be an artefact.

Incorporation of radioactivity into ferulic acid and caffeic acid could not be detected.

Table 3.7.2 Radioactivity [dpm] measured in spots attributed to capsaicin and blanks scraped off TLC plates after the separation of phenolic compounds and visualised with iodine fumes. The blanks were taken from the top left corner (above the first solvent front) of the corresponding TLC plates. The elicitation times indicated (0-24 h) are identical with the [^{14}C]phenylalanine labelling periods, except for controls (0 h) that were labelled for 24 h in the absence of fungal elicitor.

	0 h	4 h	8 h	12 h	24 h
blank	67	35	45	43	36
capsaicin	47	43	76	57	46

3.7.1.1.2 Analysis of phenolic compounds by HPLC

Samples (10 μ l) of the phenolic extracts from culture medium were subjected to HPLC as described in section 2.12.5 except that the addition of non-radioactive free phenolic standards was omitted. Fractions were collected as they were eluted from the column and analysed for radioactivity using LSC. Each fraction consisted of the column eluent of 1 min resulting in 30 fractions collected per HPLC run. The HPLC traces and the amounts of radioactivity measured in the corresponding fractions are presented in Figs. 3.7.2 to 3.7.6.

3.7.1.1.2.1 Phenolic compounds absorbing UV

The absolute amounts of phenolic compounds that absorbed UV light at 280 nm in the culture medium were relatively small compared to those found in the cells (section 3.7.1.2.2). The highest OD₂₈₀ measured was 31.8 milliabsorbance units (mAU) for phenolic 4 (Fig. 3.7.2) in the medium extract from control cultures.

The HPLC trace obtained from extracts of untreated cultures (Fig. 3.7.2) shows a number of peaks which, in varying quantity, were also detected in extracts from elicited cultures. None of the compounds could be positively identified as any of the proposed free phenolic intermediates of capsaicin biosynthesis. However, a small peak in medium extracts from 8 h elicited cultures co-eluted with cinnamic acid when the sample was spiked with free phenolic standards (Fig. 3.7.4.1). The positive identification of cinnamate was not possible, because the amount present was too small to obtain a UV spectrum and [¹⁴C]cinnamic acid was not detected by 2D-TLC. The UV spectra (240-354 nm) of the major peaks are shown in Fig. 3.7.2. The signals from smaller peaks were too weak to allow this type of analysis.

The UV spectrum of phenolic 1 (Fig. 3.7.2) with its two maxima (273 nm and 305 nm) is typical of 3,4-dioxybenzaldehyde. The retention time (R_f) of 6.4 min is close to that of protocatechuic acid. The absolute amount of the compound detected as peak 1 decreased following elicitation as estimated by its absorbance at 280 nm.

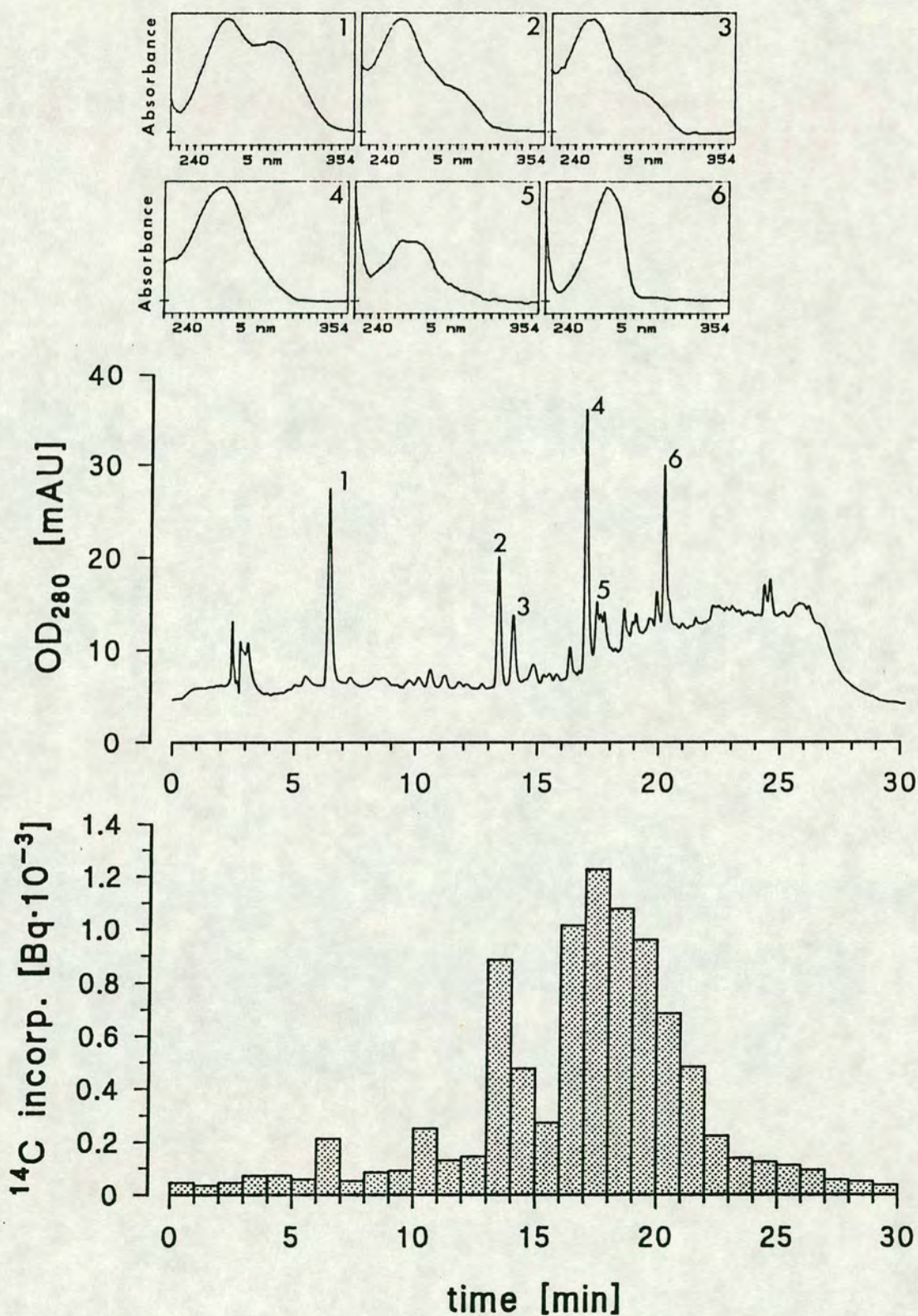


Figure 3.7.2 Phenolic compounds in diethyl ether:ethylacetate extracts obtained from the medium of control cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1 to 6) are shown above the HPLC trace. The amounts of radioactivity [Bq · 10⁻³] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

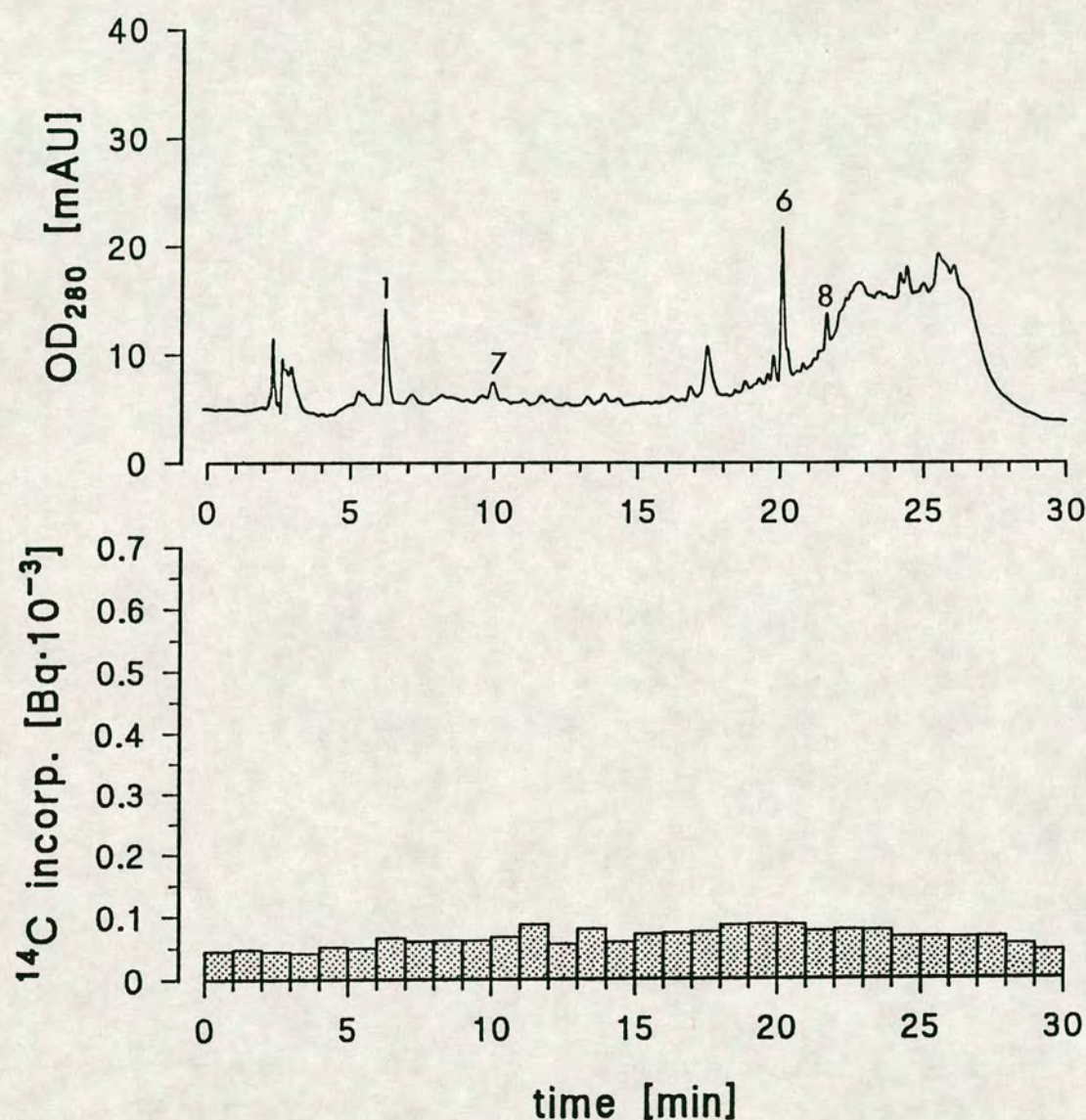


Figure 3.7.3 Phenolic compounds in diethyl ether:ethylacetate extracts obtained from the medium of 4 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks are shown in Fig. 3.7.2 (phenolic 1,6), Fig. 3.7.4 (phenolic 7), and Fig. 3.7.6 (phenolic 8). The amounts of radioactivity [Bq · 10⁻³] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

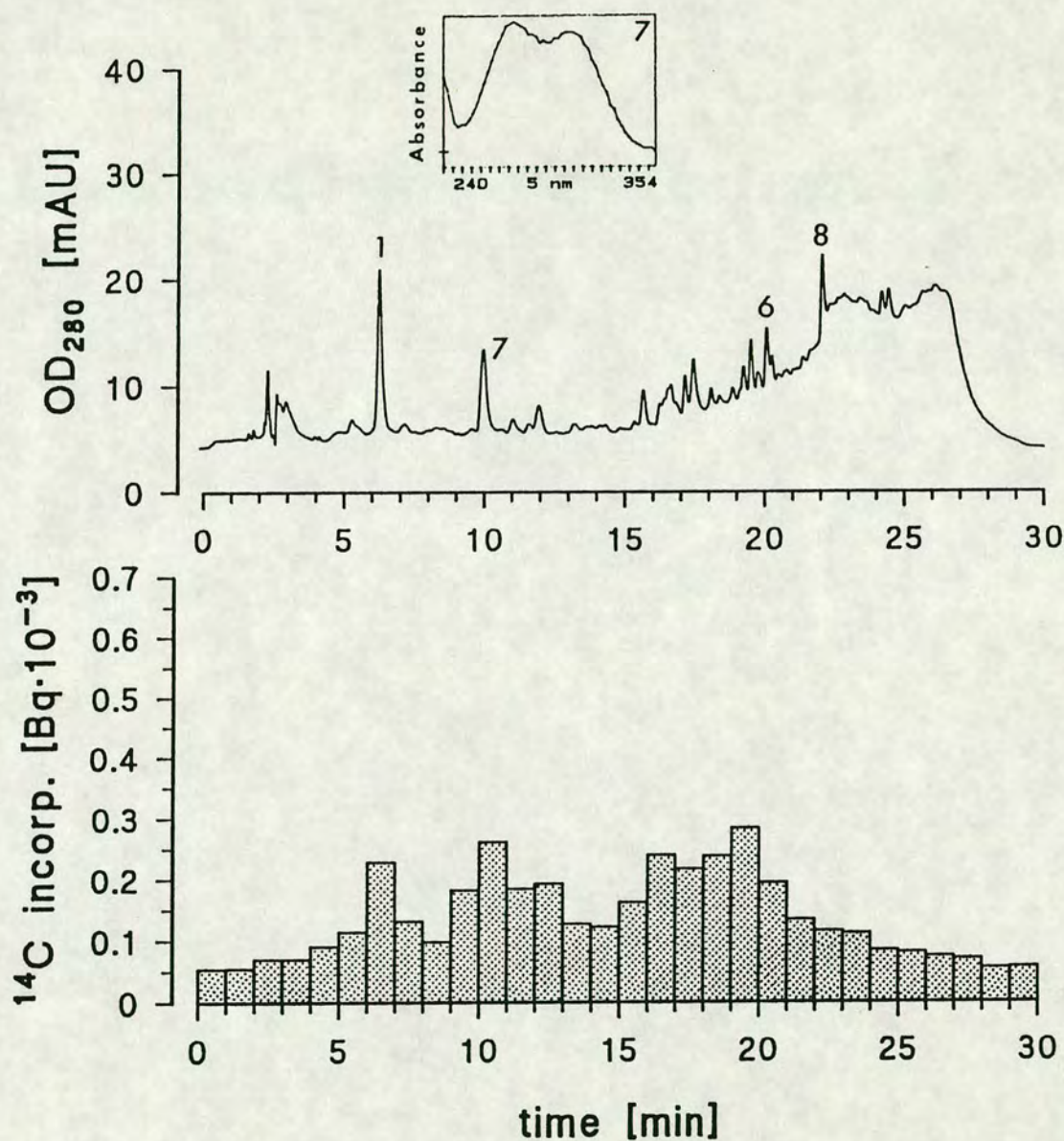


Figure 3.7.4 Phenolic compounds in diethyl ether:ethylacetate extracts obtained from the medium of 8 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectrum (240-354 nm) of phenolic 7 is shown above the HPLC trace. The UV spectra of the other major peaks are shown in Fig. 3.7.2 (phenolic 1,6), and Fig. 3.7.6 (phenolic 8). The amounts of radioactivity [Bq · 10⁻³] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

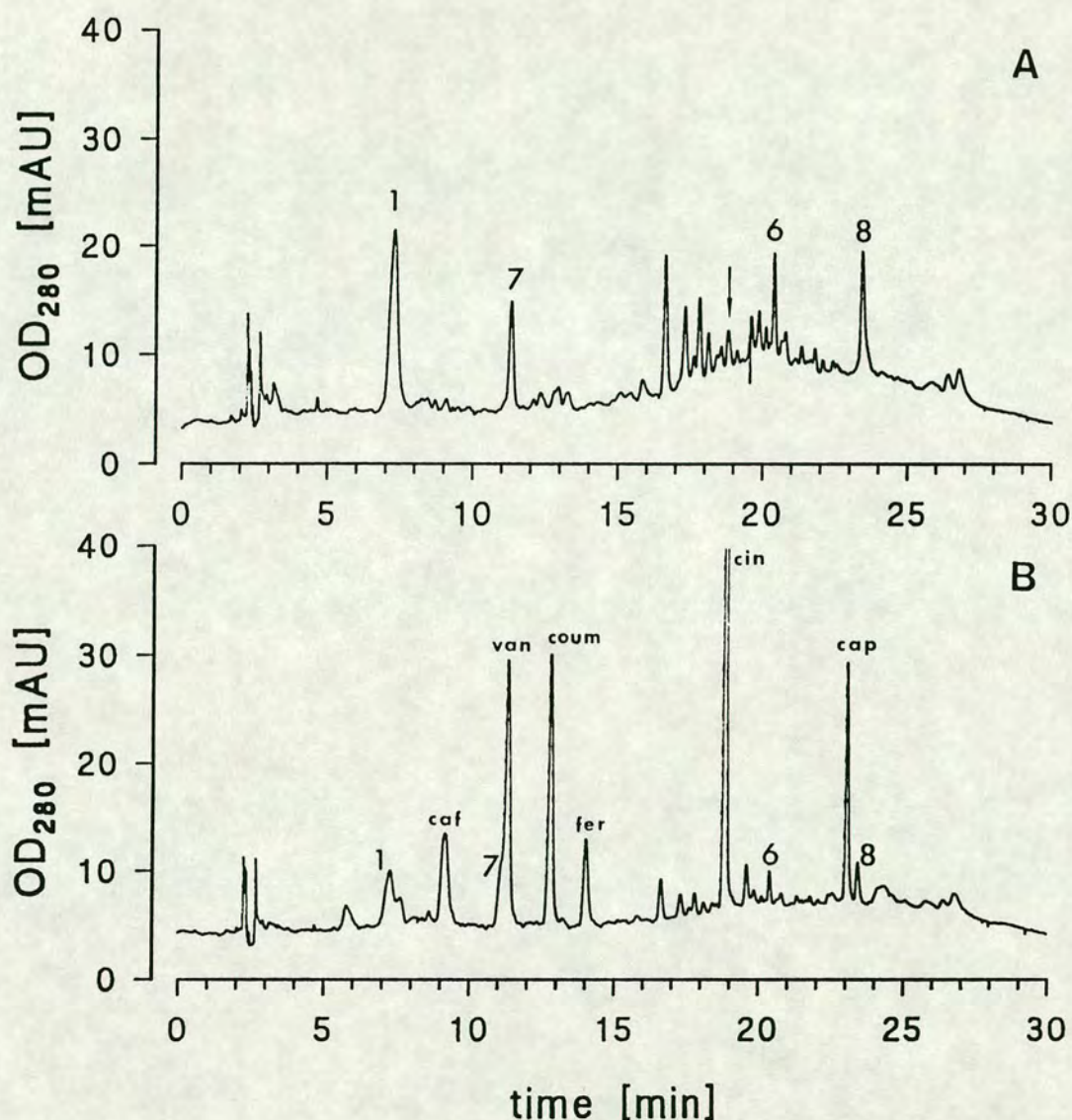


Figure 3.7.4.1 (A) The phenolic extract from the medium of 8 h elicited cultures (Fig. 3.7.4) was separated by HPLC using a new column of the same type as in Fig. 3.7.4. The UV spectra of the numbered peaks are shown in Figs. 3.7.2, 3.7.4, and 3.7.6. (B) A sample of the same extract was spiked with free phenolic standards (caf = caffeic acid, cap = capsaicin, cin = cinnamic acid, coum = coumaric acid, fer = ferulic acid, van = vanillin). The UV spectra of the standards are shown in Fig. 3.7.16. The absorbances of the numbered compounds are reduced due to the relative dilution following the addition of the standards. Phenolic 7 constitutes a small shoulder in the vanillin peak, the arrow in (A) indicates a compound with the same R_t as cinnamic acid.

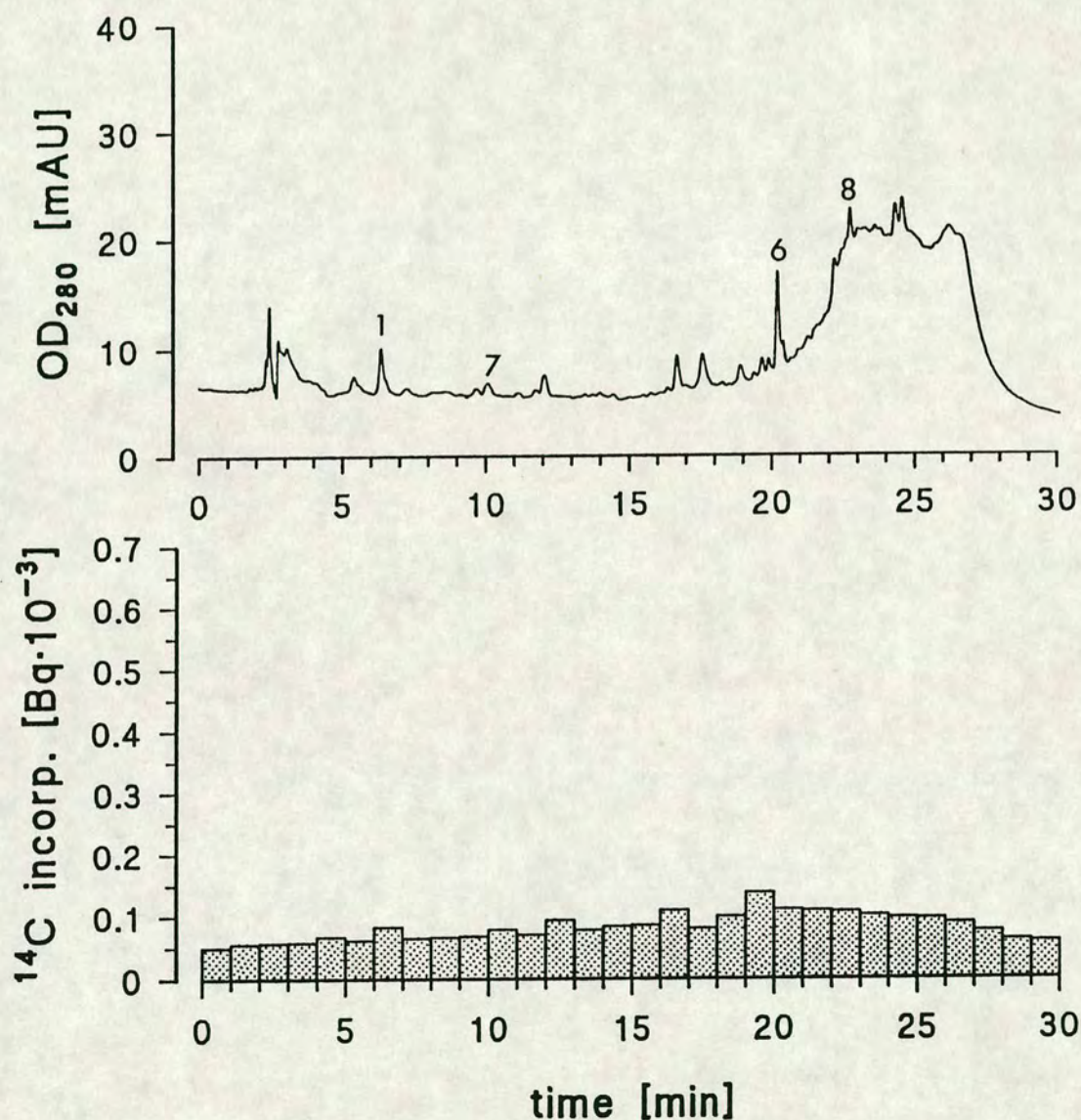


Figure 3.7.5 Phenolic compounds in diethyl ether:ethylacetate extracts obtained from the medium of 12 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks are shown in Fig. 3.7.2 (phenolic 1,6), Fig. 3.7.4 (phenolic 7), and Fig. 3.7.6 (phenolic 8). The amounts of radioactivity [$Bq \cdot 10^{-3}$] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

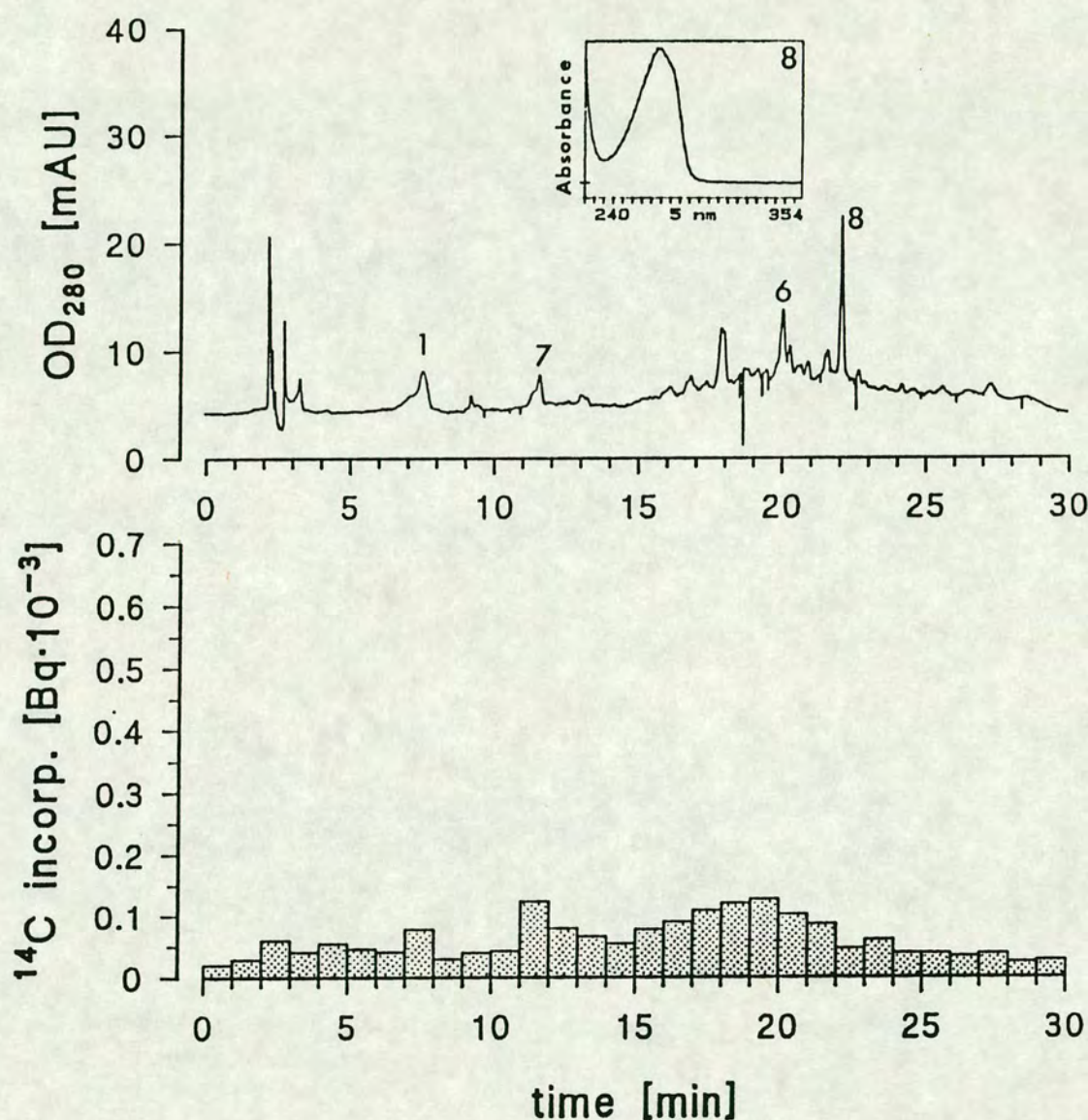


Figure 3.7.6 Phenolic compounds in diethyl ether:ethylacetate extracts obtained from the medium of 24 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectrum (240-354 nm) of phenolic 8 is shown above the HPLC trace. The UV spectra of the other major peaks are shown in Fig. 3.7.2 (phenolic 1,6), and Fig. 3.7.4 (phenolic 7). The amounts of radioactivity [Bq · 10⁻³] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

The amount of radioactivity that co-eluted with phenolic 1 (fraction 6-7 min) was similar for controls and 8 h elicited cultures (Table 3.7.3), but much lower in the other elicitation variants. The total amounts of radioactivity detected in the medium of cultures elicited for 4 h, 12 h, and 24 h was markedly lower than those in the medium of control cultures and cultures elicited for 8 h (Table 3.7.1).

Phenolics 2 and 3 (Fig. 3.7.2) have a similar type of UV spectrum similar to the UV spectrum of *m*-coumaric acid. However, this compound is rarely found in higher plants (Sukrasno, 1991). Phenolics 2 and 3 were not detected in the medium of elicited cultures (Fig. 3.7.3 - Fig. 3.7.6).

Phenolics 4 and 5 (Fig. 3.7.2) were present in high quantity in untreated cultures as estimated by their OD₂₈₀ but could not be detected in elicited cultures. The signals at the corresponding R_t in elicited cultures were too weak to obtain UV spectra.

Phenolic 6 (Fig. 3.7.2) was present in all medium extracts. The UV spectrum of this compound is similar to that of capsaicin (Fig. 2.12.1), but the R_t is *ca.* 2 min less than the R_t observed for capsaicin standards.

HPLC traces of elicited cultures showed a peak at R_t 22.1 min that was most prominent in 24 h elicited cultures (phenolic 8, Fig. 3.7.6). The UV spectrum of this compound was identical with that of capsaicin (Fig. 3.7.16). The R_t of phenolic 8 differed from that of capsaicin by only 40 sec (Fig. 3.7.4.1). Taken together, UV spectrum and R_t suggest phenolic 8 to be a compound very similar to capsaicin, probably another "capsaicinoid" like homocapsaicin. The amount of radioactivity measured in the corresponding fraction (R_t 22-23 min, Table 3.7.3) however, did not reflect the appearance and increase in amount of this compound. Hall *et al.* (1987) found that even at the height of capsaicin synthesis in *Capsicum* fruits the amount of radioactivity which found its way into capsaicin was only a very small proportion of the total amount added.

Table 3.7.3 Radioactivity [$\text{Bq} \cdot 10^{-3}$] detected in HPLC fractions obtained from di-ethyl ether:ethylacetate (1:1) extracts of culture medium. The data points correspond to the histograms shown in Figs. 3.7.2 to 3.7.6 and therefore represent single values.

R_t [min]	Radioactivity [$\text{Bq} \cdot 10^{-3}$]				
	0 h	4 h	8 h	12 h	24 h
0- 1	0.046	0.045	0.054	0.050	0.020
1- 2	0.035	0.048	0.055	0.056	0.029
2- 3	0.046	0.044	0.070	0.056	0.060
3- 4	0.073	0.042	0.070	0.058	0.041
4- 5	0.073	0.052	0.091	0.068	0.055
5- 6	0.058	0.050	0.114	0.062	0.046
6- 7	0.214	0.066	0.229	0.083	0.041
7- 8	0.053	0.061	0.132	0.065	0.078
8- 9	0.085	0.062	0.098	0.067	0.030
9-10	0.092	0.062	0.183	0.068	0.040
10-11	0.252	0.068	0.263	0.079	0.043
11-12	0.131	0.088	0.185	0.071	0.125
12-13	0.146	0.056	0.193	0.094	0.080
13-14	0.888	0.080	0.128	0.078	0.066
14-15	0.478	0.059	0.123	0.084	0.054
15-16	0.273	0.072	0.163	0.086	0.078
16-17	1.019	0.073	0.241	0.110	0.090
17-18	1.233	0.075	0.218	0.081	0.109
18-19	1.083	0.086	0.239	0.100	0.121
19-20	0.965	0.088	0.285	0.138	0.128
20-21	0.688	0.087	0.195	0.111	0.103
21-22	0.484	0.076	0.134	0.109	0.086
22-23	0.223	0.078	0.116	0.108	0.046
23-24	0.140	0.078	0.112	0.102	0.060
24-25	0.125	0.067	0.084	0.098	0.039
25-26	0.113	0.067	0.081	0.097	0.038
26-27	0.094	0.066	0.074	0.089	0.034
27-28	0.057	0.067	0.070	0.076	0.038
28-29	0.051	0.055	0.055	0.062	0.025
29-30	0.037	0.045	0.057	0.059	0.028
total	9.255	1.963	4.112	2.465	1.831

The UV spectrum of phenolic 7 (Fig. 3.7.4) could only be obtained from 8 h elicited cultures, the signal being too weak in the other medium extracts. UV spectrum and R_t of this compound are similar to vanillin or *iso*-vanillin. Phenolic 7 was eluted as a small shoulder in the vanillin peak when the sample was spiked with free phenolic standards.

3.7.1.1.2.2 Radioactivity associated with HPLC fractions

The amounts of radioactivity measured in the fractions collected from the HPLC column are plotted as histograms underneath the corresponding HPLC trace in Fig. 3.7.2 to Fig. 3.7.6. A fraction size of 1 min was chosen for practical reasons. However, the collection of 30 fractions over a 30 min HPLC run proved insufficient to allow a precise correlation of detected peaks and collected fractions. A number of smaller peaks was collected in a single 1 min fraction and radioactivity associated with broad peaks was sometimes distributed over more than one fraction.

Untreated cultures

Four maxima of radioactivity were detected in the fractions collected from untreated (control) cultures (Fig. 3.7.2). The major amount of radioactivity was associated with the fractions 16-22 min, containing phenolics 4-6. High amounts of radiolabel were also found in the fractions containing phenolic 2 and 3 (13-15 min), phenolic 1 (6-7 min), and in fraction 10-11 min, corresponding to the R_t of phenolic 7 (Fig. 3.7.4).

Elicited cultures

Fractions collected from elicited cultures contained considerably less radioactivity than those from untreated cultures (Table 3.7.3). Differences between the fractions were less pronounced compared to controls. In cultures elicited for 4 h (Fig. 3.7.3) and 12 h (Fig. 3.7.5) radioactivity was almost evenly distributed over all 30 fractions. Activity measured in these fractions was just above background level. The histograms of

the fractions collected from cultures elicited for 8 h (Fig. 3.7.4) and 24 h (Fig. 3.7.6) showed three areas of high radioactivity. These were the fractions 16-21 min and the fractions containing phenolic 1 and phenolic 7.

The two major differences in the distribution of radioactivity in elicited cultures compared to controls were as follows.

- (1) The absolute amounts of radioactivity measured were lower in extracts from elicited cultures.
- (2) Phenolics 2 and 3 and the amount of radioactivity co-eluted with these compounds were absent in extracts from elicited cultures.

3.7.1.2 Extracts from cultured cells

Phenolic compounds were extracted from cultured cells as described in section 2.12.3. Methanol extracts, without liquid-liquid extraction into diethyl ether, were subjected to analysis by HPLC. Water soluble compounds in the methanol extracts however, created problems during TLC separation (see below) with the solvent systems used. Therefore, the methanol extracts were dried *in vacuo* and the phenolic compounds were extracted into diethyl ether as described in section 2.12.3.

3.7.1.2.1 Analysis of phenolic compounds by TLC

Samples of the phenolic extracts from cultured cells were analysed using 2D-TLC. The TLC plates were loaded with 30 μ l sample and 20 μ l non-radioactive standards (section 3.7.1.1.1) and eluted using benzene:acetic acid (9:2, 1st dimension) and *iso*-propanol:butanol:ammonia:water (6:2:1:1, 2nd dimension) as described in section 2.12.4. Upon drying, the plates were subjected to direct autoradiography (section 2.11.1) and exposed for 21 d. Following autoradiography, the phenolic compounds were visualised as described in section 2.12.4 using 1% $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ and 0.5% $\text{K}_3\text{Fe}(\text{CN})_6$. A second set of TLC plates was prepared as described in section 3.7.1.1.1 for the estimation of radiolabel incorporated into free phenolic compounds. Iodine

stained (section 3.7.1.1.1) spots of phenolic standards were scraped off the TLC plates and radioactivity was measured using LSC.

Fig. 3.7.7 shows the result of the autoradiographic detection of the radioactively labelled phenolic compounds in methanol extracts that were separated by 2D-TLC. Radioactive compounds that comigrated with non-radioactive standards are labelled as described in the figure legend. Radioactive spots without a label denote unidentified phenolic compounds. The non-radioactive standards migrated in the normal manner, but the radioactive compounds in the methanol extracts did not separate in the first solvent system. The faint spots detected on the autoradiograph of 8 h elicited cultures (Fig. 3.7.7) were disregarded, because the migration in the first dimension seemed generally inhibited. It is likely, that the final positions of these radioactive compounds on the TLC plate were distorted and the apparent comigration with the non-radioactive standards was possibly coincidental. The reasons for the poor separation of radioactive phenolic compounds in the non-polar solvent system are not fully understood, but the phenomenon is possibly due to the presence of water soluble (polar) phenolic glycosides in the methanolic extracts (Sukrasno, personal communication). Non-radioactive free phenolic standards were not mixed with the samples but loaded on top of the radioactive samples onto the TLC plates thus, resulting in the unhindered separation of the standards. In order to alleviate the problem caused by phenolic glycosides, the methanolic extracts were dried *in vacuo* and free phenolics were extracted into diethyl ether as described in section 2.12.3. Possible losses during this treatment are acknowledged in the analysis of the results.

Samples of the diethyl ether extracts were subjected to 2D-TLC and analysed as above. The separation of the radioactive phenolic compounds was considerably improved and seemed typical. The results of the autoradiographic analysis of the 2D-TLC separations are presented in Fig. 3.7.8.

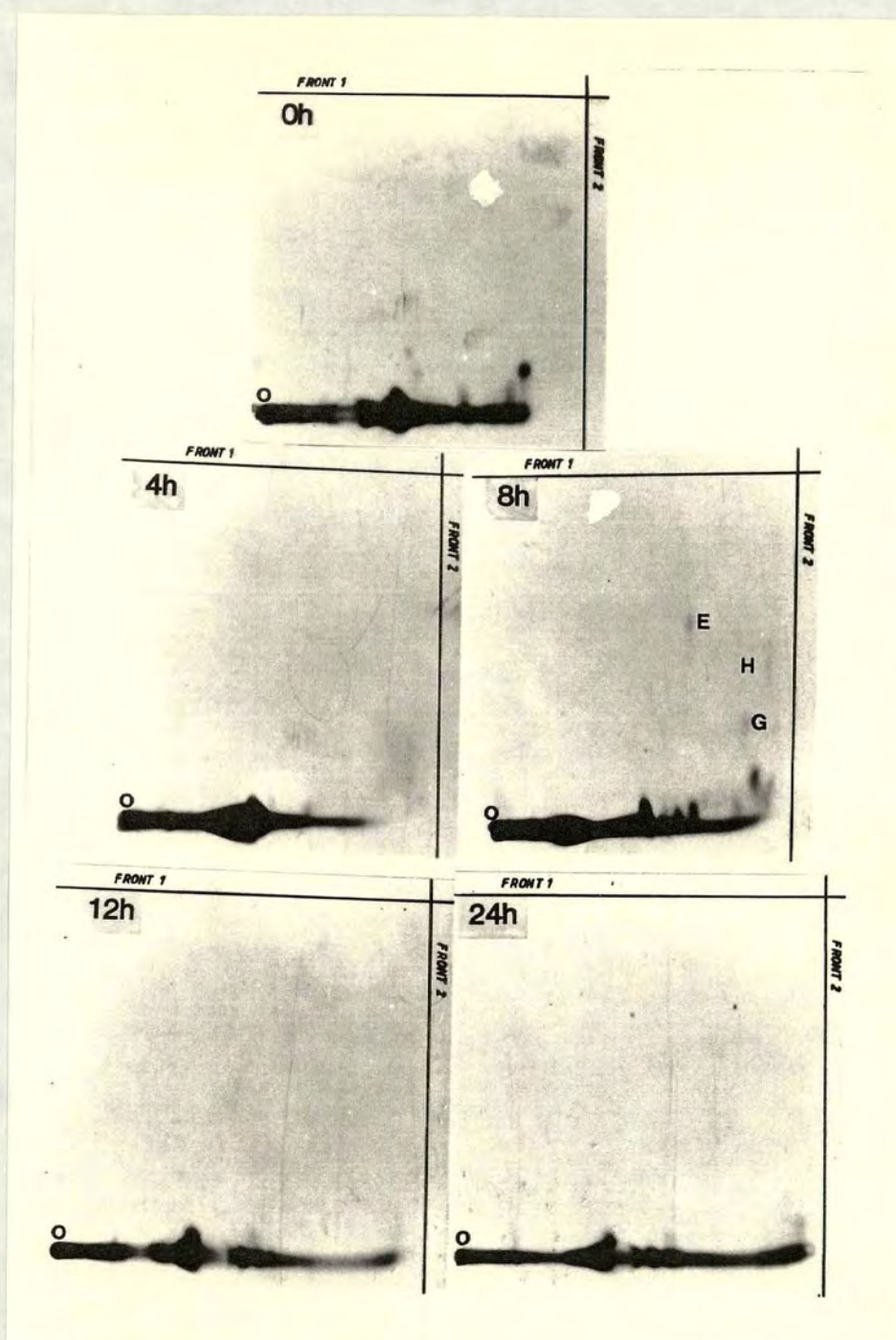


Figure 3.7.7 Phenolic compounds extracted into methanol from cultured cells separated by 2D-TLC and analysed for [^{14}C] incorporation using direct autoradiography. The elicitation times indicated in the top left corner of each autoradiograph were identical with the labelling periods, except for controls (0 h) which were labelled for 24 h in the absence of fungal elicitor. The solvent fronts are marked at the top (benzene:acetic acid, 9:2) and at the right hand side (*iso*-propanol: butanol: ammonia: water, 6:2:1:1) of each autoradiograph. The starting point (origin = O) of the separation is situated in the bottom left corner of each autoradiograph. Radioactive compounds that comigrated with non-radioactive standards are labelled as follows. A = phenylalanine (see text), B = cinnamic acid, C = coumaric acid, D = ferulic acid, E = vanillin, F = vanillylamine, G = vanillyl alcohol, H = capsaicin.

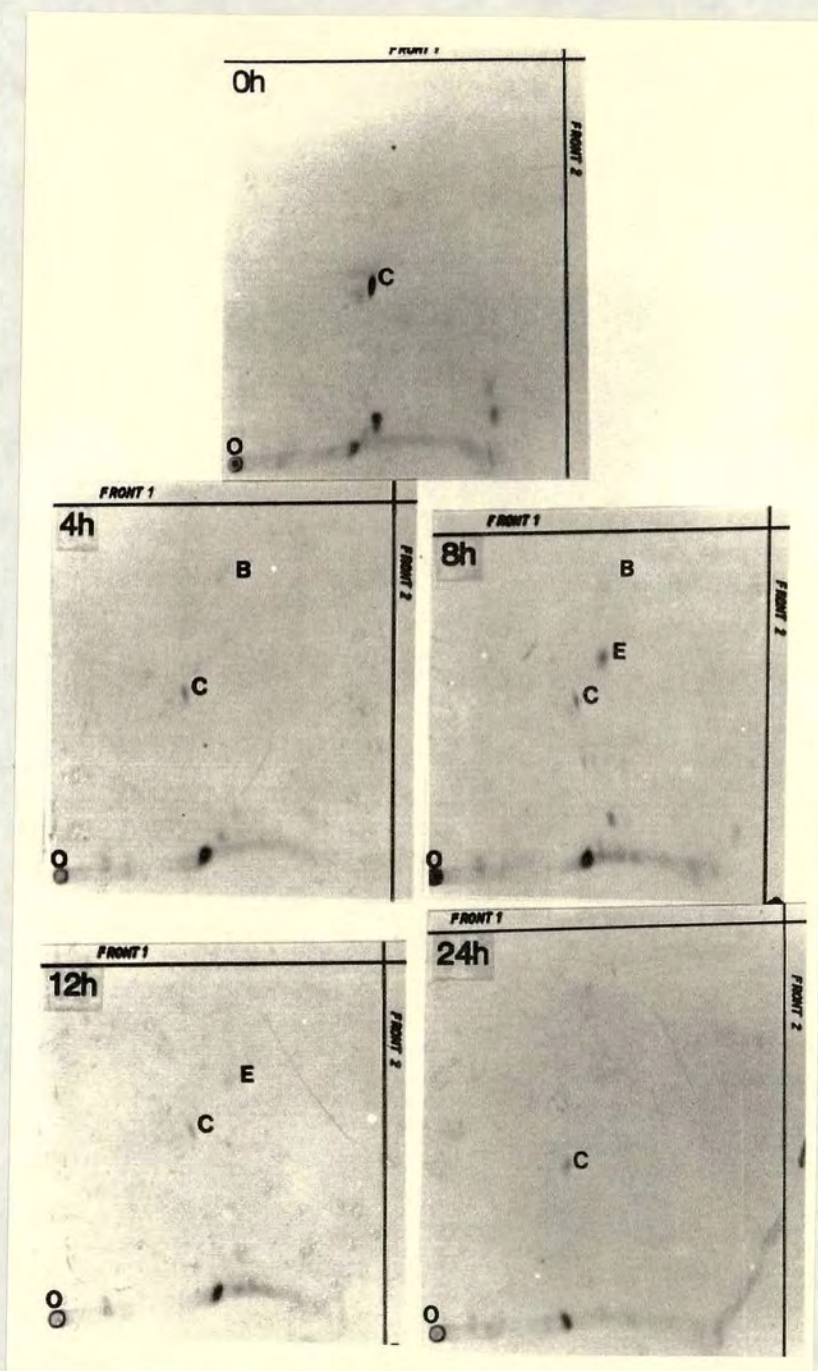


Figure 3.7.8 Phenolic compounds extracted into diethyl ether from methanolic extracts of cultured cells separated by 2D-TLC and analysed for [^{14}C] incorporation using direct autoradiography. The elicitation times indicated in the top left corner of each autoradiograph were identical with the labelling periods, except for controls (0 h) which were labelled for 24 h in the absence of fungal elicitor. The solvent fronts are marked at the top (benzene:acetic acid, 9:2) and at the right hand side (*iso*-propanol:butanol:ammonia:water, 6:2:1:1) of each autoradiograph. The starting point (origin = O) of the separation is situated in the bottom left corner of each autoradiograph. Radioactive compounds that comigrated with non-radioactive standards are labelled as follows. A = phenylalanine (see text), B = cinnamic acid, C = coumaric acid, D = ferulic acid, E = vanillin, F = vanillylamine, G = vanillyl alcohol, H = capsaicin.

[¹⁴C]Coumaric acid (label C, Fig. 3.7.8) was detected in all cell extracts. It was the only free phenolic intermediate of the proposed biosynthetic pathway leading to capsaicin detected in untreated cultures. Incorporation of radiolabel into [¹⁴C]coumaric acid seemed to be highest in controls, but quantification was omitted for the reasons stated above.

[¹⁴C]Cinnamic acid (label B, Fig. 3.7.8), the product of PAL activity, appeared following elicitation of *Capsicum* cell cultures. It was detected in extracts of 4 h and 8 h elicited cultures, but was not detected at later stages of the elicitor treatment.

[¹⁴C]Vanillin (label E, Fig. 3.7.8) was detected in 8 h elicited cultures and a very faint spot was visible in 12 h elicited cultures. In agreement with the results obtained from medium extracts, [¹⁴C]vanillin was not detected in cultures elicited for 24 h. Incorporation of radiolabel was not detected in ferulic and caffeic acid.

3.7.1.2.2 Analysis of phenolic compounds by HPLC

Samples (10 µl) of the methanolic extracts from cultured cells were subjected to HPLC as described in section 2.12.5 except that the addition of non-radioactive free phenolic standards was omitted. Fractions were collected as they were eluted from the column and analysed for radioactivity using LSC. Each fraction consisted of the column eluent of 1 min resulting in 30 fractions collected per HPLC separation. The HPLC traces and the radioactivity measured in the corresponding fractions are presented in Fig. 3.7.9 to Fig. 3.7.13. The UV spectra of the major phenolic compounds detected by their absorption at 280 nm are shown in Fig. 3.7.11.

3.7.1.2.2.1 Phenolic compounds absorbing UV

The methanol extracts from cultured cells contained a greater number and higher amounts of phenolic compounds than the diethyl ether extracts from culture medium. This was possibly due to the presence of a number of water soluble phenolic glycosides in the methanolic extracts (section 3.7.1.2.1). The highest OD₂₈₀ measured in

cell extracts was 143.3 mAU compared to 31.8 mAU in medium extracts. Treatment with fungal elicitor transiently increased the amount of phenolic compounds in methanol extracts in general, and of compound 3 (Fig. 3.7.11) in particular. The highest absorbance measured in each HPLC separation is listed in Table 3.7.4. The amount of phenolic compounds in the methanolic extracts increased sharply after the introduction of the fungal elicitor and reached a maximum after 8 h (Table 3.7.4).

In addition to an overall increase in phenolics, a selective increase in phenolic 3 was observed. The UV spectrum of phenolic 3 (Fig. 3.7.11) shows that this compound does not absorb strongly at 280 nm, unlike phenolic 6,7. It can be concluded, that the large increase in OD₂₈₀ observed for phenolic 3 resulted from an even stronger increase in absolute amount. The OD₂₈₀ of phenolic 3 (143.3 mAU) was higher than the OD₂₈₀ of phenolic 6,7 (*ca.* 133 mAU) in 8 h elicited cultures. In all other cultures the absorbance of phenolic 6,7 was highest.

Table 3.7.4 A comparison of the highest absorbances (OD₂₈₀) measured during HPLC analysis of methanolic extracts from cultured *Capsicum* cells.

Elicitation period	OD ₂₈₀ [mAU]	compound
0 h	67.4	phenolics 6,7 and 8
4 h	115.3	phenolics 6,7
8 h	143.3	phenolic 3
12 h	77.0	phenolics 6,7
24 h	62.1	phenolics 6,7

The increase in absolute amount of phenolic compounds in the cell extracts was transient and had returned to control levels after 24 h (Table 3.7.4). The general increase in phenolic compounds and the specific increase in phenolic 3 coincided with the observed rise and fall in PAL activity (section 3.4.3).

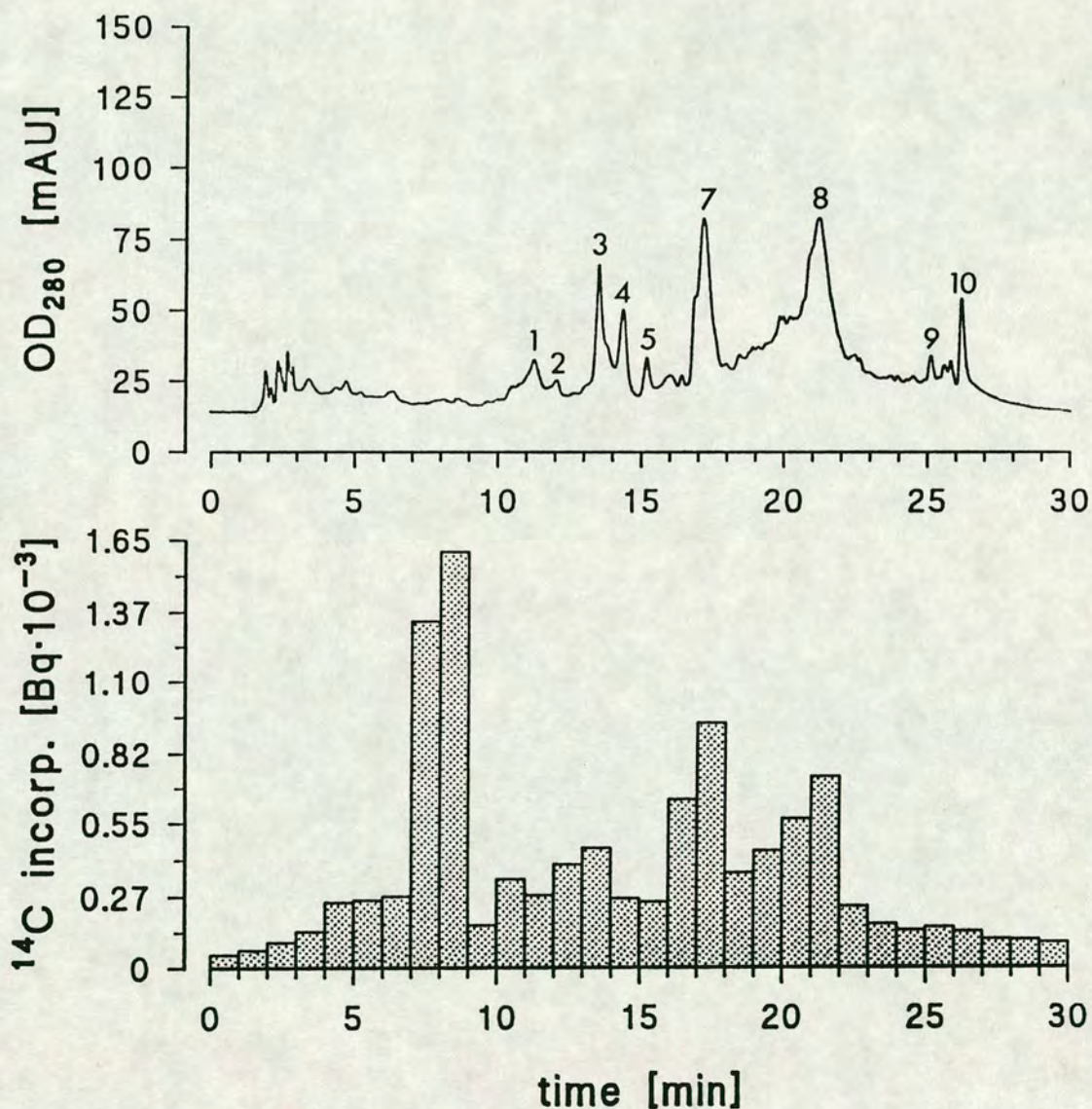


Figure 3.7.9 Phenolic compounds in methanol extracts obtained from cells of untreated cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-10) are shown in Fig. 3.7.11. The amounts of radioactivity [Bq · 10⁻³] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

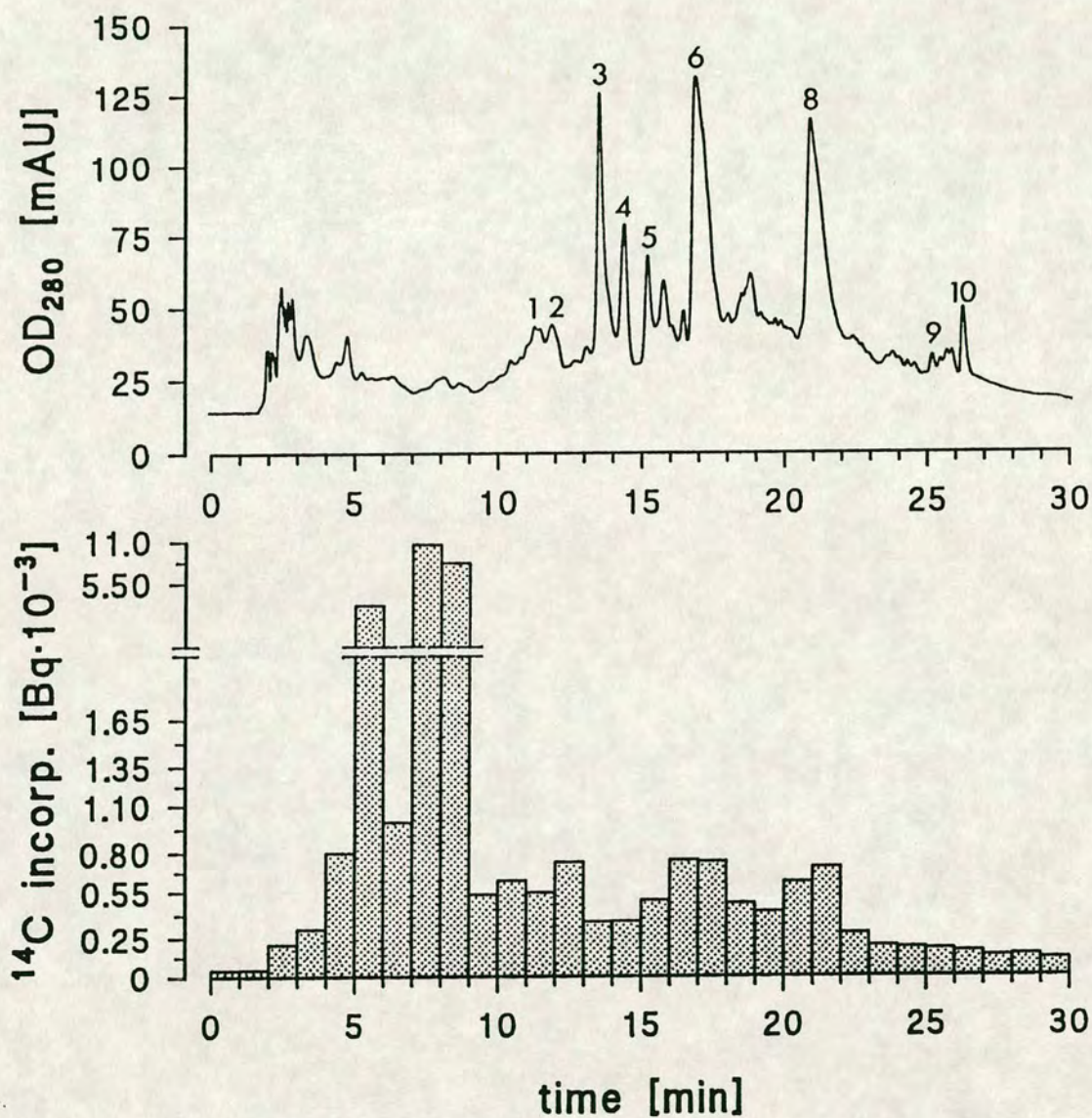


Figure 3.7.10 Phenolic compounds in methanol extracts obtained from cells of 4 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-10) are shown in Fig. 3.7.11. The amounts of radioactivity [$Bq \cdot 10^{-3}$] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

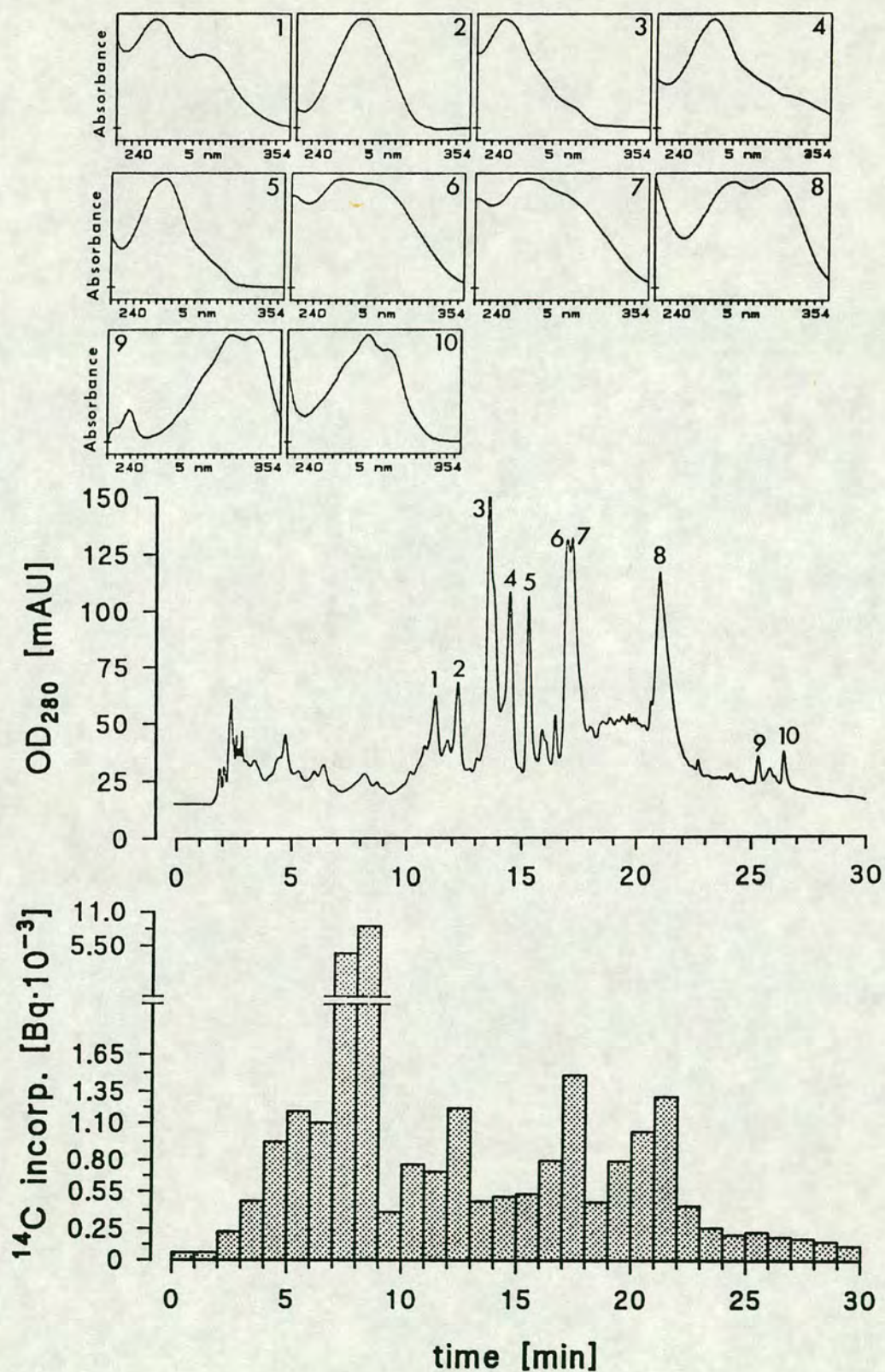


Figure 3.7.11 Phenolic compounds in methanol extracts obtained from cells of 8 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-10) are shown above the HPLC trace. The amounts of radioactivity [Bq · 10⁻³] detected in the fractions collected from the HPLC column are plotted as histograms underneath the HPLC trace.

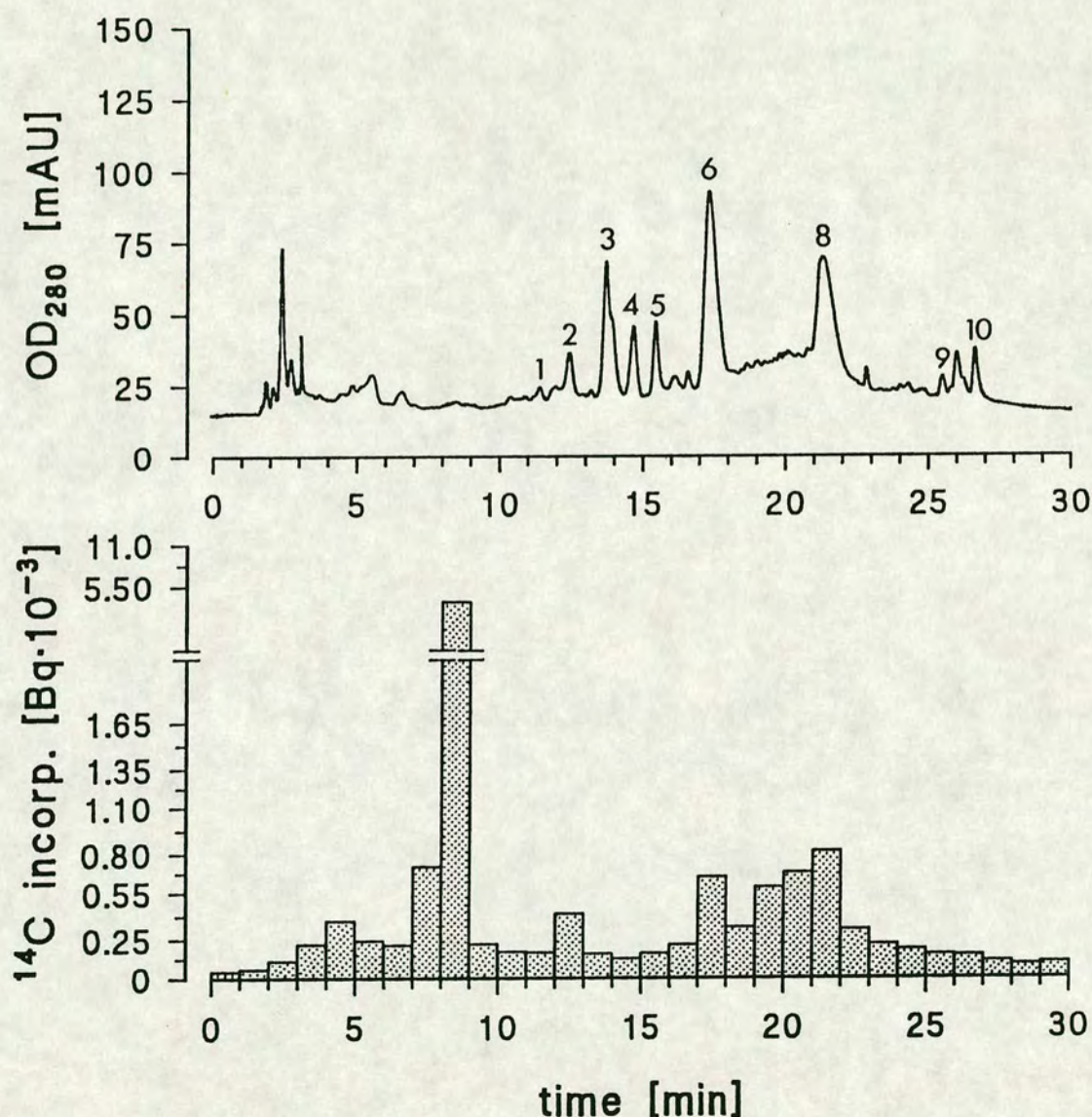


Figure 3.7.12 Phenolic compounds in methanol extracts obtained from cells of 12 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-10) are shown in Fig. 3.7.11. The amounts of radioactivity [$Bq \cdot 10^{-3}$] detected in the fractions collected from the HPLC column are plotted as histogram underneath the HPLC trace.

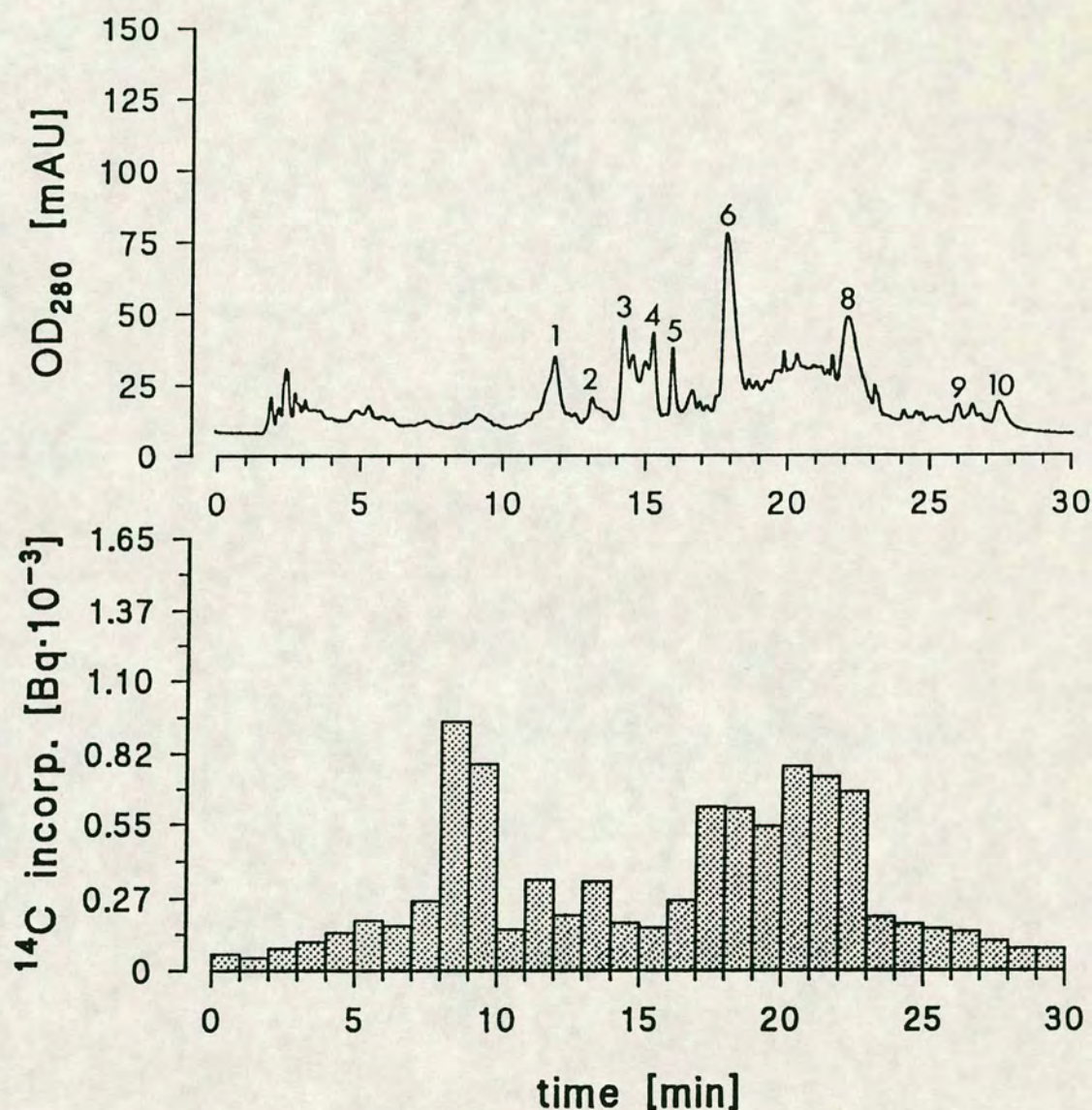


Figure 3.7.13 Phenolic compounds in methanol extracts obtained from cells of 24 h elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-10) are shown in Fig. 3.7.11. The amounts of radioactivity [Bq · 10⁻³] detected in the fractions collected from the HPLC column are plotted as histogram underneath the HPLC trace.

Very little information is available about the phenolics detected in the cell extracts. None of the phenolic compounds could be identified as free phenolic intermediate of the capsaicin biosynthetic pathway.

The UV spectrum of phenolic 2 is typical for *p*-hydroxybenzaldehyde. R_f and UV spectrum of phenolic 3 were very similar to those observed for phenolic 3 in medium extracts from untreated cultures. This compound could not be detected in medium extracts from elicited cultures. The UV spectrum and the R_f of phenolic 8 (Fig. 3.7.11) were identical to those of the phenolic compound tentatively identified as 3,4-dimethoxycinnamoyl glycoside by Sukrasno (1991).

3.7.1.2.2.2 Radioactivity associated with HPLC fractions

The amounts of radioactivity measured in the fractions collected from the HPLC column are plotted as histograms underneath the corresponding HPLC trace in Fig. 3.7.9 to Fig. 3.7.13.

The distribution pattern of radiolabel among the HPLC fractions collected from control cultures (Fig. 3.7.9) and cultures elicited for 24 h (Fig. 3.7.13) was very similar. Both cultures were labelled for 24 h using 5 μ Ci [14 C]phenylalanine. Shorter labelling periods (4 h, 8 h, and 12 h) resulted in an accumulation of radioactivity in the fractions at R_f 5-9 min (Table 3.7.5). These relatively large amounts of radioactivity did not seem to be associated with phenolic compounds that absorb UV at 280 nm. A likely explanation is, that the radiolabel detected in these fractions is unincorporated [14 C]phenylalanine. Phenylalanine is eluted during the early (polar) part of the methanol:acetic acid gradient and its OD₂₈₀ is practically zero (Fig. 2.5.1).

Apart from the high levels of radioactivity in fractions 5-9 min (see above), maximum amounts of radiolabel were detected in the fractions containing phenolic 2, phenolics 6,7, and phenolic 8. Treatment with fungal elicitor increased the amounts of radioactivity detected in these fractions (Table 3.7.5).

Table 3.7.5 Radioactivity [$\text{Bq} \cdot 10^{-3}$] detected in HPLC fractions obtained from methanol extracts of cultured cells.

R_t [min]	Radioactivity [$\text{Bq} \cdot 10^{-3}$]				
	0 h	4 h	8 h	12 h	24 h
0- 1	0.050	0.045	0.059	0.045	0.061
1- 2	0.068	0.047	0.061	0.059	0.048
2- 3	0.098	0.021	0.228	0.113	0.083
3- 4	0.139	0.308	0.476	0.221	0.105
4- 5	0.248	0.800	0.952	0.371	0.142
5- 6	0.258	2.717	1.195	0.244	0.188
6- 7	0.272	0.998	1.106	0.216	0.168
7- 8	1.335	10.687	4.399	0.722	0.262
8- 9	1.603	8.273	8.795	3.646	0.947
9-10	0.163	0.534	0.388	0.223	0.783
10-11	0.338	0.625	0.770	0.173	0.154
11-12	0.277	0.548	0.712	0.168	0.342
12-13	0.349	0.741	1.221	0.418	0.208
13-14	0.458	0.355	0.476	0.160	0.336
14-15	0.264	0.358	0.513	0.128	0.179
15-16	0.251	0.497	0.536	0.163	0.161
16-17	0.643	0.754	0.802	0.217	0.265
17-18	0.940	0.745	1.489	0.655	0.619
18-19	0.362	0.476	0.470	0.329	0.613
19-20	0.447	0.421	0.798	0.589	0.545
20-21	0.568	0.616	1.036	0.683	0.777
21-22	0.733	0.709	1.314	0.819	0.738
22-23	0.233	0.279	0.440	0.315	0.678
23-24	0.166	0.198	0.262	0.221	0.203
24-25	0.143	0.187	0.205	0.188	0.177
25-26	0.153	0.177	0.228	0.154	0.158
26-27	0.138	0.161	0.188	0.149	0.148
27-28	0.108	0.131	0.174	0.111	0.113
28-29	0.106	0.138	0.151	0.089	0.085
29-30	0.095	0.114	0.117	0.103	0.084
total	11.006	32.660	29.561	11.692	9.370

The amount of radioactivity that co-eluted with phenolic 2 was specifically increased following elicitation and reached the levels of phenolics 6,7 and phenolic 8 after 8 h elicitation. The elicitation effect was transient and the amounts of radioactivity returned to control levels after 24 h. Radioactivity that co-eluted with phenolic 3, the compound that was specifically induced during elicitor treatment (section 3.7.1.2.2.1), was not increased following elicitation.

3.7.2 Long term effects of elicitation on phenylpropanoid metabolism

The observed changes in phenylpropanoid metabolism following elicitation of *Capsicum frutescens* cell cultures (section 3.7.1) were transient and the amount of free phenolic intermediates returned to control levels within 24 h. However, the observed transient changes may induce changes in phenylpropanoids that are not immediately detectable. Therefore, long term effects on phenylpropanoid metabolism have been studied. The expression "long term" denotes the time after the described transient changes have faded, i.e. > 24h. An exemplary elicitation period of 72 h was chosen to study long term effects of fungal elicitation on phenylpropanoid metabolism in *Capsicum* cell cultures.

Fungal elicitor preparation and 10 μ Ci [14 C]phenylalanine were added simultaneously to the experimental flasks. Control cultures were incubated with 10 μ Ci [14 C]phenylalanine in the absence of fungal elicitor for 72 h before harvest. Phenolic compounds were extracted from the culture medium and cultured cells as described in section 2.12.2 and section 2.12.3. In order to detect soluble phenolic compounds released into the medium and retained in cultured cells simultaneously, the ethereal extract from cultured cells was combined with the chloroform extract from the culture medium, dried *in vacuo*, and redissolved in 1 ml HPLC grade methanol for analysis.

Analysis by TLC and HPLC was performed as described in section 2.12.4 and section 2.12.5, respectively.

3.7.2.1 Analysis of phenolic compounds by TLC

Samples of the phenolic extracts from *Capsicum* cultures were subjected to 2D-TLC as described in section 3.7.1.2.1. Fig. 3.7.14 shows two autoradiographs of extracts from elicited and non-elicited cultures separated by 2D-TLC.

Two different free phenolic intermediates of capsaicin biosynthesis were identified by their comigration with added non-radioactive standards in controls (0 h, Fig. 3.7.14) and elicited cultures (72 h, Fig. 3.7.14). [^{14}C]cinnamic acid (label B, Fig. 3.7.14) and [^{14}C]coumaric acid (label C, Fig. 3.7.14) were detected in control cultures. In elicited cultures [^{14}C]coumaric acid and [^{14}C]vanillin (label E, Fig. 3.7.14) were identified. The two major radioactive spots at the bottom of each autoradiograph, that did not migrate in the non-polar solvent system, are possibly phenolic glycosides (Sukrasno, personal communication). Their presence is possibly due to contamination of the diethyl ether phase with the aqueous phase during liquid-liquid extraction, a common technical problem (Sukrasno, unpublished observation). The composition of this glycoside fraction seemed to be affected by elicitation (Fig. 3.7.14).

3.7.2.2 Analysis of phenolic compounds by HPLC

Samples (20 μl) of the phenolic extracts from *Capsicum* cultures were subjected to HPLC analysis as described in section 2.12.5. The solvent gradient (acetic acid:methanol) used for the 30 min HPLC separation is shown in Fig. 3.7.15. Fractions were collected as they were eluted from the column and analysed for radioactivity using LSC. Each fraction consisted of the column eluent of 1 min, resulting in 30 fractions collected per HPLC separation. The HPLC traces and the amount of radioactivity measured in the corresponding fractions are presented in Fig. 3.7.16 and Fig. 3.7.17. The UV spectra of the major phenolic compounds detected are shown in Fig. 3.7.16.

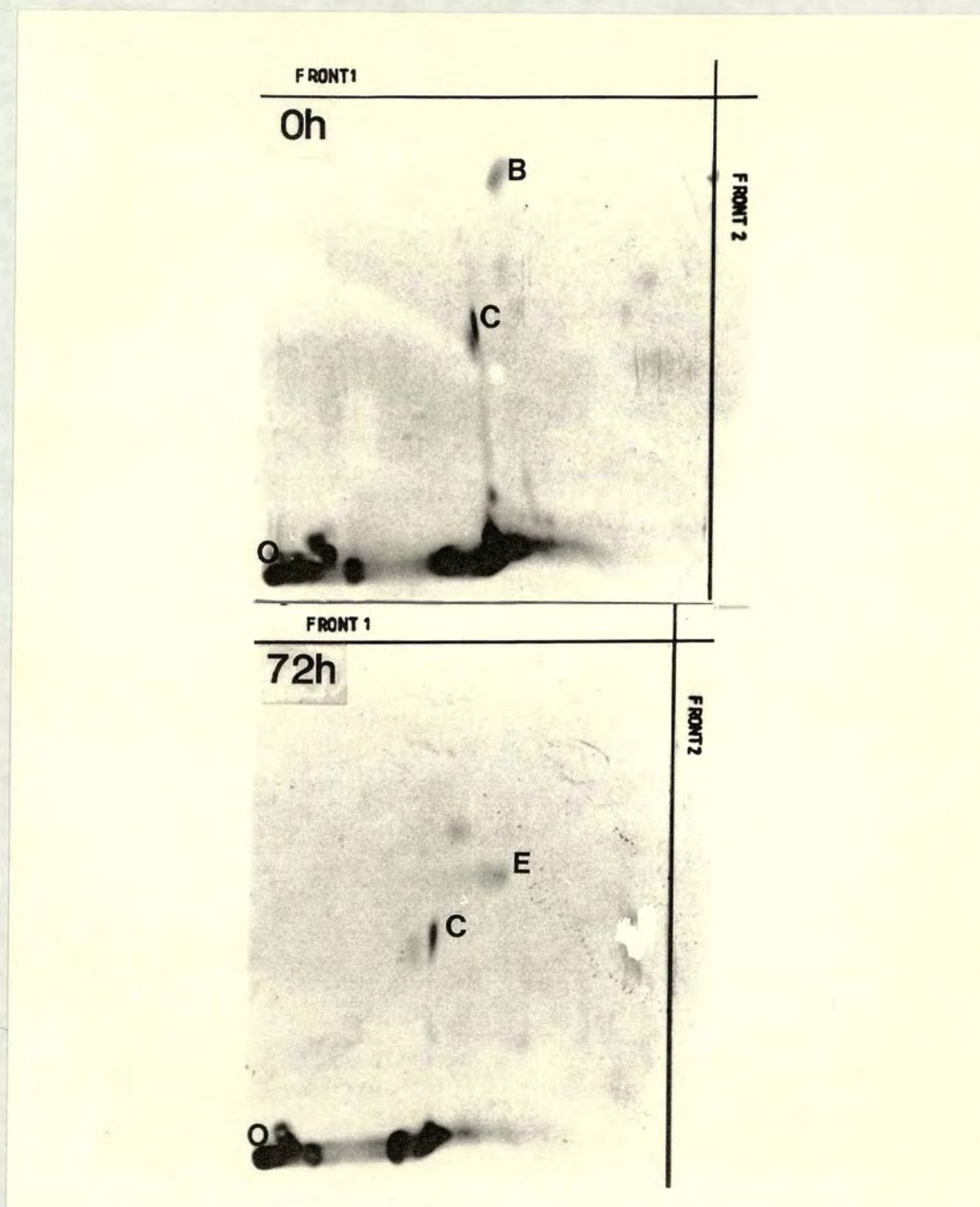


Figure 3.7.14 Phenolic compounds extracted from elicited cultures (72 h) and controls (0 h) separated by 2D-TLC and analysed for [^{14}C] incorporation using direct autoradiography. The solvent fronts are marked at the top (benzene:acetic acid, 9:2) and at the right hand side (*iso*-propanol:butanol:ammonia: water, 6:2:1:1) of each autoradiograph. The starting point (origin = O) of the separation is situated in the bottom left corner of each autoradiograph. Radioactive compounds that comigrated with non-radioactive standards are labelled as follows. A = phenylalanine (see text), B = cinnamic acid, C = coumaric acid, D = ferulic acid, E = vanillin, F = vanillylamine, G = vanillyl alcohol, H = capsaicin.

The phenolic compounds detected by their OD₂₈₀ are all free phenolic standards that were added during the extraction procedure (sections 2.12.2, 2.12.3) except phenolic 6 (Fig. 3.7.16). The UV spectrum of phenolic 6 is identical to that of vanillic acid, thus indicating an aromatic moiety of similar structure. Its R_f close to that of capsaicin, indicates a more non-polar compound than free vanillic acid. Phenolic 6 was detected in elicited cultures (Fig. 3.7.17) and controls (Fig. 3.7.16).

Vanillylamine, added as a non-radioactive standard during extraction, was not extracted into the organic phase but remained in the aqueous phase.

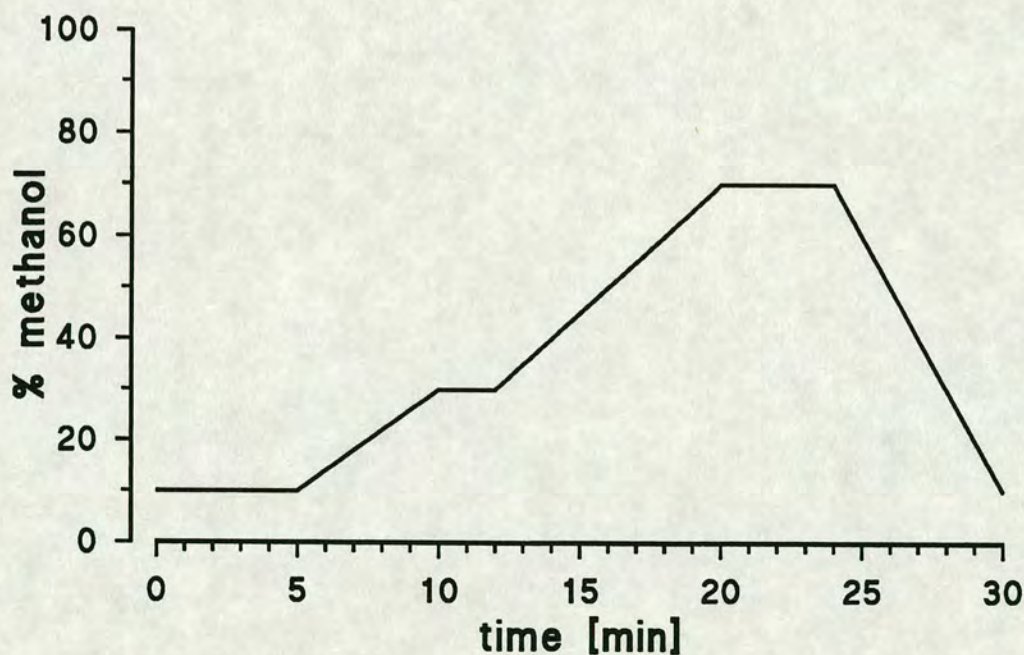


Figure 3.7.15 Phenolic compounds analysed by HPLC using a methanol:acetic acid gradient. The relative methanol concentration [%] in the solvent mixture is plotted against time [min].

Large amounts of radioactivity were eluted with caffeic acid (8-10 min, Fig. 3.7.16). 2D-TLC analysis (section 3.7.2.1) however, showed that [¹⁴C]caffeic acid was not present in the phenolic extracts. Peak 1 has a shoulder that indicates the elution of

more than one compound with similar R_t . The amount of radioactivity detected in fractions 8-10 min was much higher in controls than in the corresponding fractions of elicited cultures. A likely explanation is a higher concentration of unincorporated [^{14}C]phenylalanine in control cultures. Apart from the difference in the amount of unincorporated label, elicited cultures and controls differed in the amount of radioactivity measured in the fraction containing phenolic 6 (Table 3.7.6). Elicitation of *Capsicum* cultures resulted in a large increase in radioactivity that co-eluted with phenolic 6.

However, peak 6 in the HPLC trace of elicited cultures (Fig. 3.7.17) exhibited a small shoulder, indicating the co-elution of another compound. Furthermore, the R_t of phenolic 6 and capsaicin were very close, resulting in both compounds being collected in the same fraction in control cultures (Fig. 3.7.16). In elicited cultures phenolic 6 and capsaicin were collected in adjacent fractions and it is possible, that the radioactivity measured in the capsaicin fraction was a spill-over from phenolic 6. Both compounds were eluted during the most non-polar part of the solvent gradient (70% methanol, Fig. 3.7.15).

Table 3.7.6 Radioactivity [$\text{Bq} \cdot 10^{-2}$] detected in HPLC fractions obtained from organic extracts of *Capsicum* cultures. Controls (0 h) and elicited cultures (72 h) were labelled for 72 h using [^{14}C]phenylalanine.

R_t [min]	Radioactivity [$\text{Bq} \cdot 10^{-2}$]			
	solvent gradient as in Fig. 3.7.15		solvent gradient as in Fig. 3.7.18	
	0 h	72 h	0 h	72 h
0- 1	0.483	0.508	0.567	0.583
1- 2	0.417	0.442	0.533	0.600
2- 3	0.633	0.775	2.142	1.775
3- 4	0.700	0.892	6.275	3.825
4- 5	0.825	0.850	4.317	2.717
5- 6	0.958	0.958	5.617	2.333
6- 7	1.583	1.575	30.225	4.267
7- 8	2.267	1.750	4.750	1.942
8- 9	18.492	3.092	1.258	1.108
9-10	15.908	0.783	0.992	0.933
10-11	1.517	1.042	0.892	0.967
11-12	1.500	1.167	0.942	1.083
12-13	2.417	0.900	0.825	0.783
13-14	1.292	0.750	1.142	0.783
14-15	0.883	1.175	1.483	2.475
15-16	1.242	0.683	1.225	1.000
16-17	0.875	0.850	2.475	4.867
17-18	1.092	2.042	1.225	1.833
18-19	1.900	1.133	0.917	0.983
19-20	1.117	1.092	0.833	0.858
20-21	0.967	1.225	0.825	0.883
21-22	1.400	5.792	0.867	0.775
22-23	2.850	1.592	0.742	0.667
23-24	1.033	0.927	0.792	0.792
24-25	0.842	0.942	0.750	0.927
25-26	0.775	0.733	0.767	0.625
26-27	0.792	0.892	0.883	0.833
27-28	0.633	0.658	0.700	0.775
28-29	0.692	0.567	0.925	0.950
29-30	0.667	0.692	0.850	0.850
total	66.752	36.479	76.736	43.792

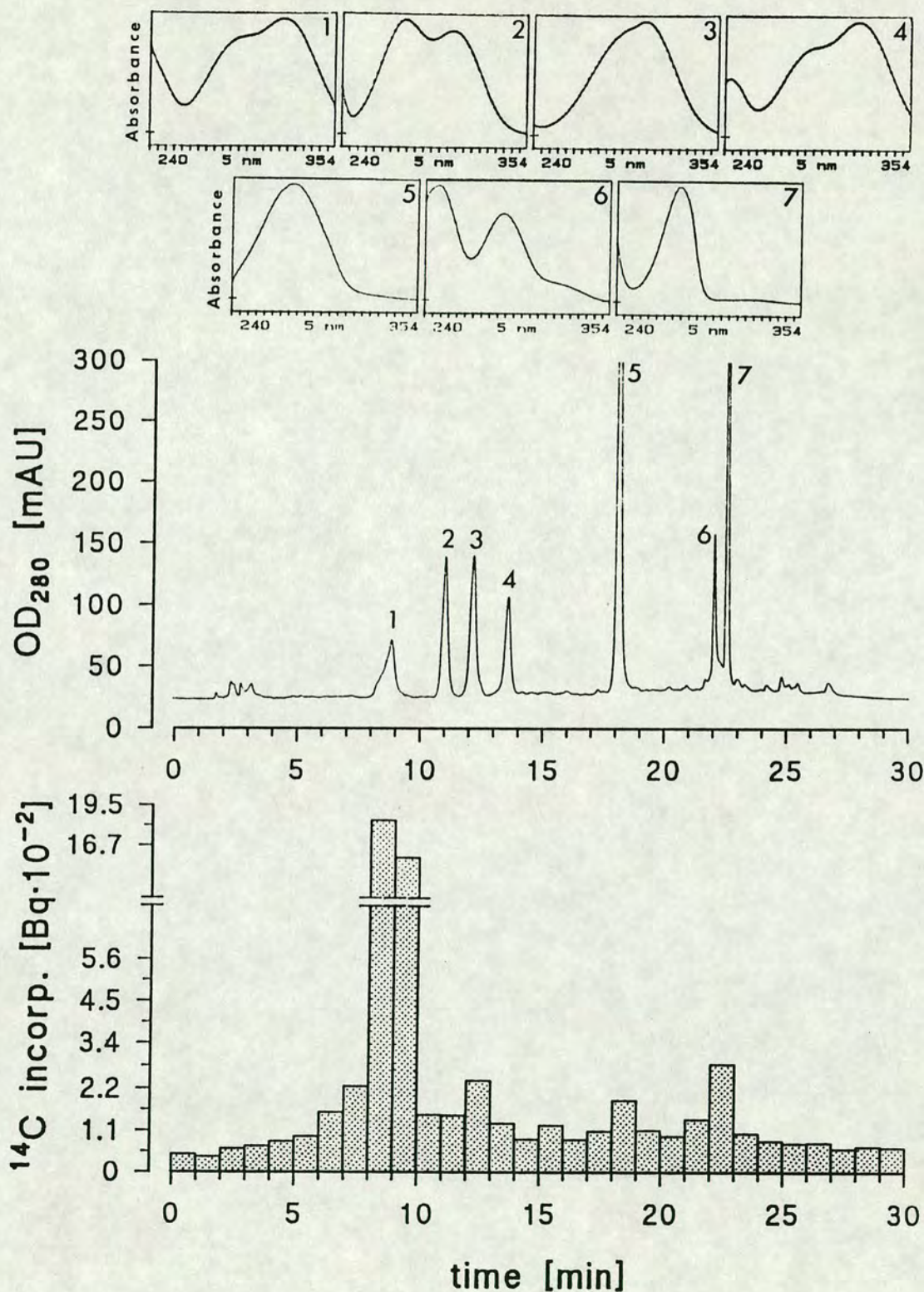


Figure 3.7.16 Phenolic compounds in organic extracts obtained from control cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-7) are shown above the HPLC trace. The amounts of radioactivity [$\text{Bq} \cdot 10^{-2}$] detected in the fractions collected from the HPLC column are plotted as histogram underneath the HPLC trace. 1 = caffeic acid, 2 = vanillin, 3 = coumaric acid, 4 = ferulic acid, 5 = cinnamic acid, 6 = unidentified compound, 7 = capsaicin.

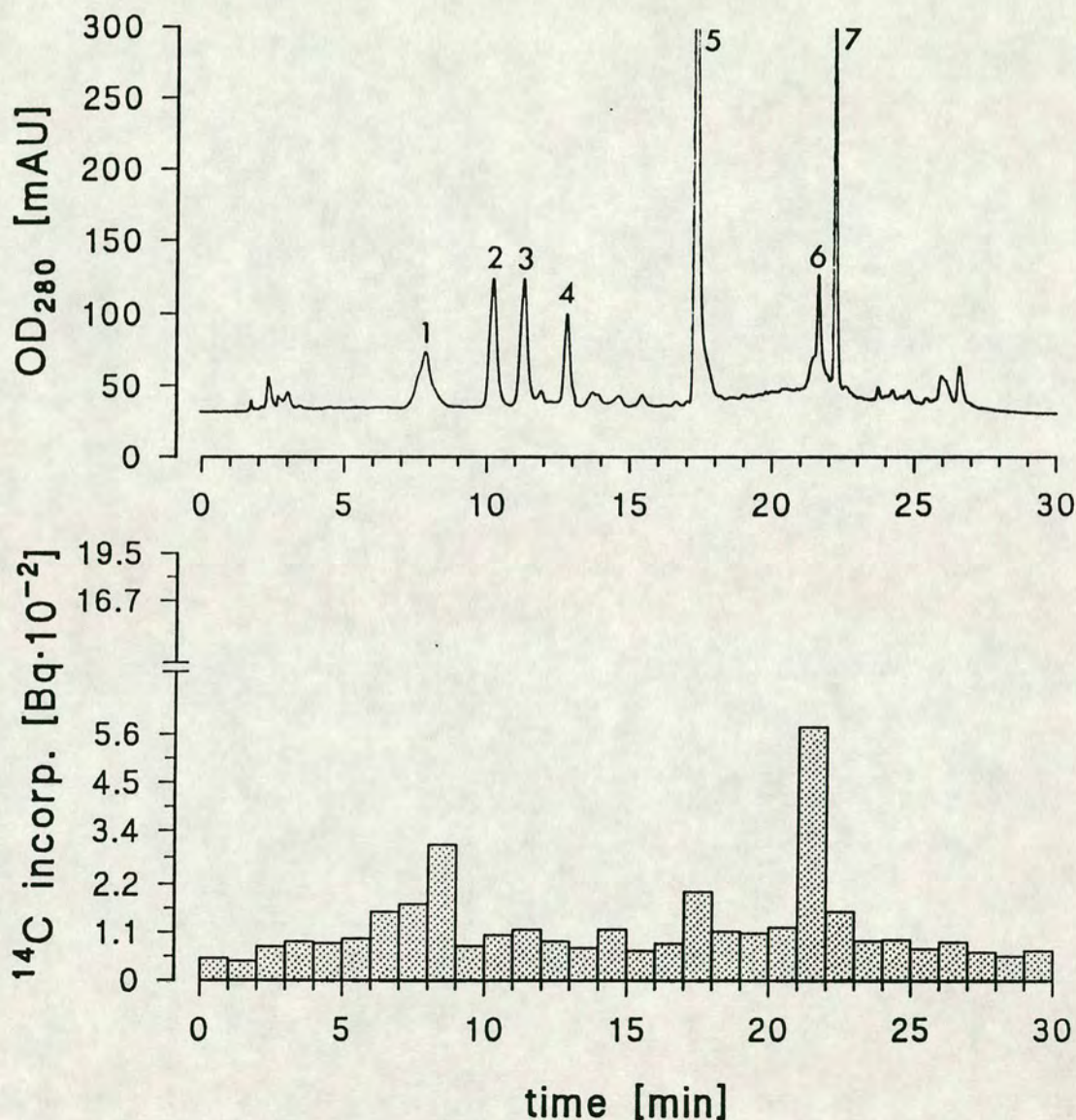


Figure 3.7.17 Phenolic compounds in organic extracts obtained from elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-7) are shown in Fig. 3.7.16. The amounts of radioactivity [Bq · 10⁻²] detected in the fractions collected from the HPLC column are plotted as histogram underneath the HPLC trace. 1 = caffeic acid, 2 = vanillin, 3 = coumaric acid, 4 = ferulic acid, 5 = cinnamic acid, 6 = unidentified compound, 7 = capsaicin.

In an attempt to achieve a better separation of capsaicin from phenolic 6 and any compound(s) that co-eluted with phenolic 6, the solvent gradient was changed. The relative methanol concentration in the acetic acid:methanol mixture was raised to 60% during the first 5 min (Fig. 3.7.18). This resulted in the elution of the majority of phenolic compounds in the early part of the HPLC separation (Fig. 3.7.19 and Fig. 3.7.20). The methanol concentration was then slowly increased to 70% (5-24 min), resulting in a clear separation of phenolic 6 and capsaicin. The shoulder of peak 6, observed during the HPLC analysis of phenolic compounds from elicited cultures (Fig. 3.7.17), was not detected when the modified solvent gradient was used (Fig. 3.7.20). Following capsaicin, a number of phenolic compounds were also eluted from the column that were not detected when the original solvent gradient was used. Most notable were two broad peaks at R_t 14-15 min and R_t 16-18 min. Both signals were too weak

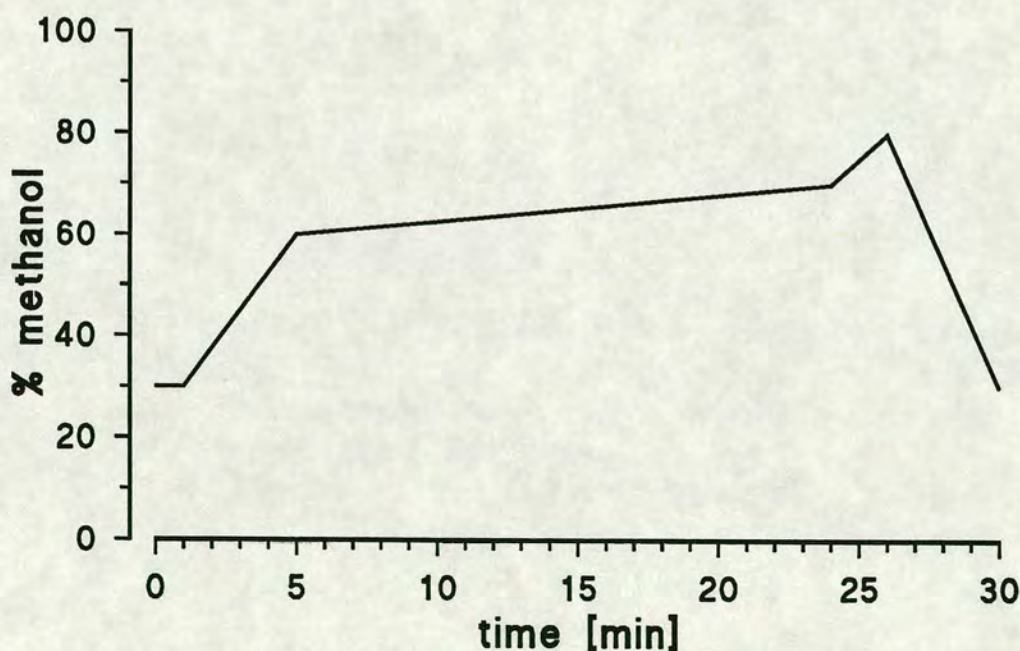


Figure 3.7.18 Phenolic compounds were analysed by HPLC using a methanol:acetic acid gradient. The relative methanol concentration [%] in the solvent mixture is plotted against time [min].

to obtain the corresponding UV spectra. The shape of the peaks suggests that several compounds contributed to each signal.

Only minor amounts of radioactivity were detected in the fraction containing phenolic 6 (Fig. 3.7.20 and Table 3.7.6). The radioactivity previously measured for phenolic 6 (Fig. 3.7.17) co-eluted with the compounds at R_t 14-15 min and 16-18 min when the modified solvent gradient was used. The amount of radioactivity that co-eluted with these compounds was increased following elicitation. It cannot be concluded that the radiolabel in co-elution with the above phenolics is necessarily incorporated into these compounds, but the results presented demonstrate that, in accordance with the results obtained from 2D-TLC analysis, elicitation of *Capsicum frutescens* cell cultures using *Gliocladium deliquescens* spore preparations did not increase the incorporation of [^{14}C]phenylalanine into capsaicin. However, considerable changes in phenylpropanoid metabolism do take place following elicitation that need further investigation.

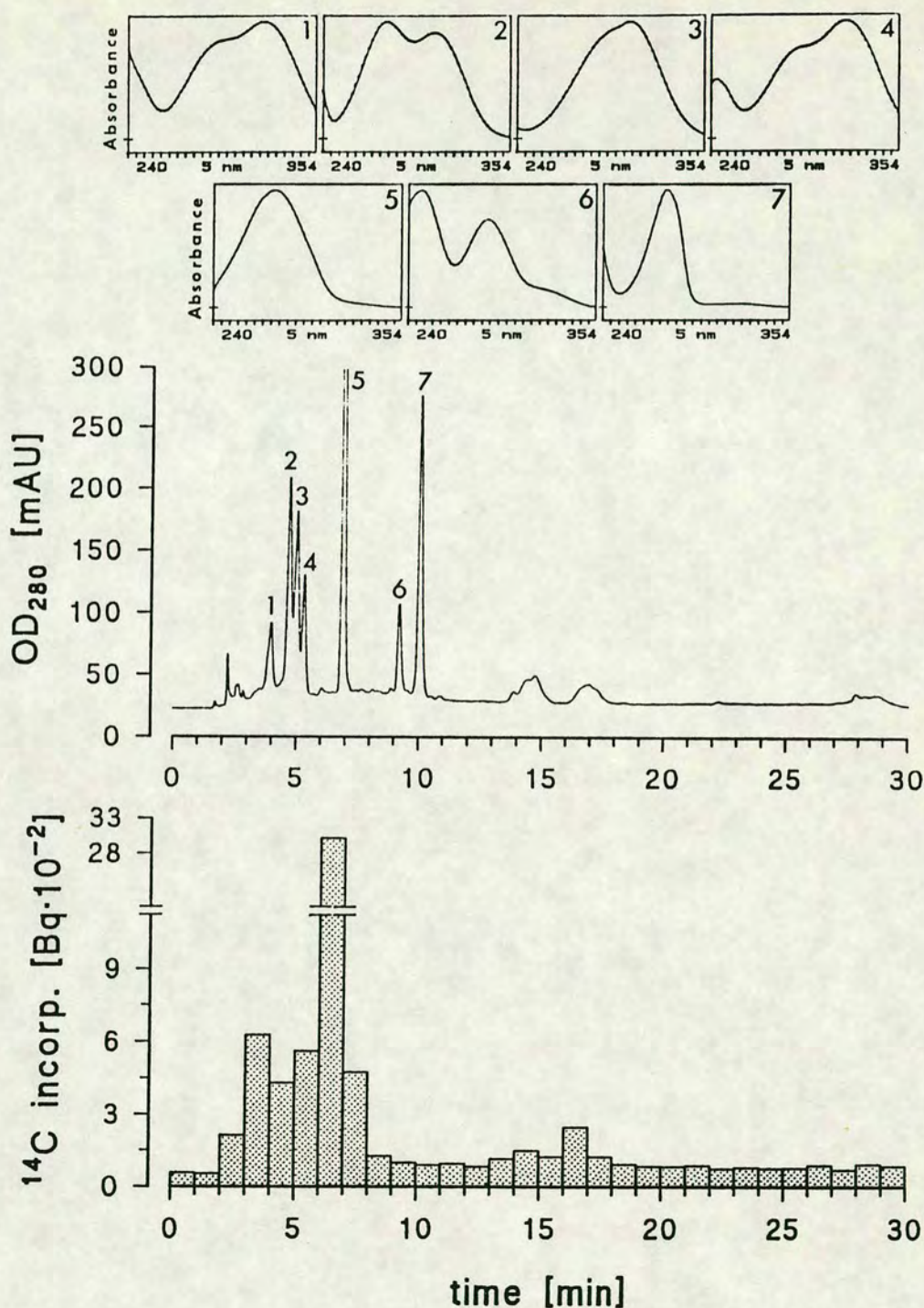


Figure 3.7.19 Phenolic compounds in organic extracts obtained from control cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-7) are shown above the HPLC trace. The amounts of radioactivity [$\text{Bq} \cdot 10^{-2}$] detected in the fractions collected from the HPLC column are plotted as histogram underneath the HPLC trace. 1 = caffeic acid, 2 = vanillin, 3 = coumaric acid, 4 = ferulic acid, 5 = cinnamic acid, 6 = unidentified compound, 7 = capsaicin.

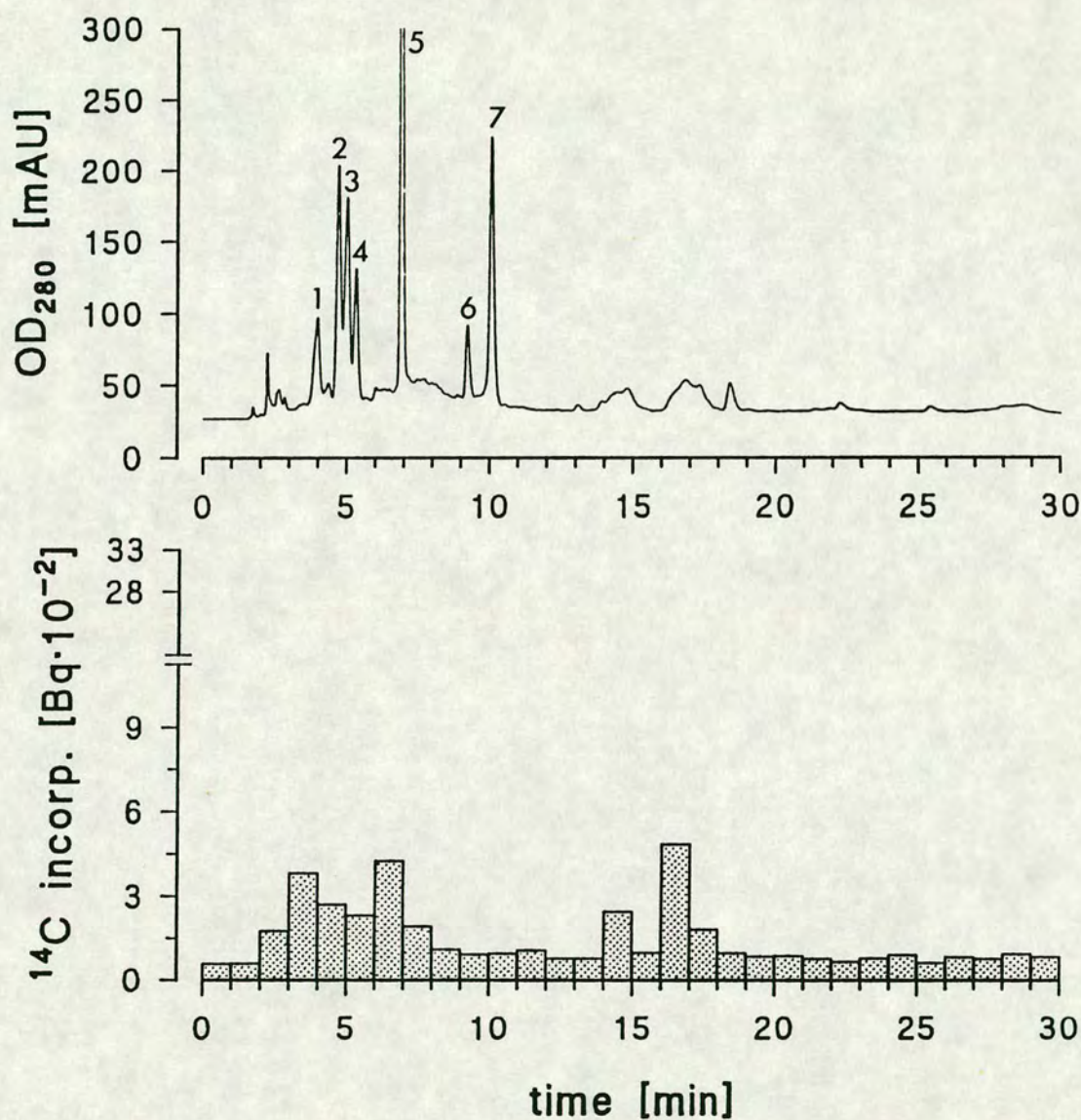


Figure 3.7.20 Phenolic compounds in organic extracts obtained from elicited cultures separated by HPLC and detected by their absorbance at 280 nm. The UV spectra (240-354 nm) of the major peaks (phenolic 1-7) are shown in Fig. 3.7.19. The amounts of radioactivity [$\text{Bq} \cdot 10^{-2}$] detected in the fractions collected from the HPLC column are plotted as histogram underneath the HPLC trace. 1 = caffeic acid, 2 = vanillin, 3 = coumaric acid, 4 = ferulic acid, 5 = cinnamic acid, 6 = unidentified compound, 7 = capsaicin.

3.7.3 Summary

The experiments described in section 3.7 were designed to study the effects of elicitation on phenylpropanoid metabolism in *Capsicum* cultures. The capsaicin biosynthetic pathway was chosen as a model system for general phenylpropanoid metabolism and attention was focussed on the synthesis of the free phenolic intermediates.

Phenylpropanoid metabolism was studied by following the incorporation of [^{14}C]phenylalanine into phenolic compounds during the observed transient induction of PAL activity (section 3.4) and after PAL activity had returned to control levels.

The majority of [^{14}C]phenylalanine was incorporated into unidentified phenolic compounds, some of these were possibly water soluble phenolic glycosides. Incorporation of [^{14}C] was detected in cinnamic acid, coumaric acid, and vanillin, supposed intermediates of capsaicin biosynthesis. The appearance of [^{14}C]cinnamic acid did not seem to be correlated with the pattern of induction observed for PAL activity (section 3.4.3). [^{14}C]coumaric acid was detected in elicited cultures and controls. Incorporation of radiolabel into vanillin was not detected in untreated cultures but was induced following elicitation. Its induction seemed to follow the pattern of PAL activity but it could also be detected after PAL activity had returned to basal levels ("long term" effect).

The absolute amount of free phenolic compounds detectable in the culture medium decreased following elicitation. The accumulation of a phenolic compound with a R_f very close to capsaicin and an UV spectrum identical to capsaicin was observed following elicitation. The amount of radioactivity that co-eluted with this compound was very small. 2D-TLC analysis indicated the presence of very small amounts of [^{14}C]capsaicin in elicited medium extracts. The amount of phenolic compounds detected in cultured cells was transiently increased.

This general increase and a specific increase of phenolic 3 coincided with the induction pattern of PAL activity described in section 3.4.3. Following this transient increase, long term elicited cultures showed a marked increase in incorporation of [^{14}C]phenylalanine into unidentified, highly non-polar phenolic compounds.

4. DISCUSSION

4.1 Introduction

Fungal elicitors have been shown to modify phenylpropanoid metabolism in a range of cell cultures (Hahlbrock and Scheel, 1989; Eilert, 1987 and references therein) including *Capsicum frutescens* (Yeoman *et al.*, 1989; Holden *et al.*, 1988a,b). Such modifications involve the synthesis of the intermediates leading to various cell wall components including lignin (Funk and Brodelius, 1990a) and in the chilli pepper to capsaicin (Holden *et al.*, 1988a). It is well known, that the enhancement of the phenylpropanoid pathway is a consequence of spectacular increases in the activity of a number of enzymes in the pathway, especially the first few, PAL, CA4H, and 4CL (for a review see Hahlbrock and Scheel, 1989).

Accordingly, the effect of fungal elicitation on phenylpropanoid metabolism in cell cultures of *Capsicum frutescens* was studied with particular attention paid to the regulation of PAL and its influence on capsaicin synthesis.

The following discussion of experimental results is divided into five major parts. Firstly (section 4.2), the suitability of capsaicin biosynthesis as a model system for secondary metabolism is considered. Secondly, the effect of elicitation on the accumulation of soluble phenolic compounds is examined (section 4.3) and then the observed effects of fungal elicitation on the regulation of PAL are discussed (section 4.4). In section 4.5 an integrated analysis of the induction of PAL activity and its effects on phenylpropanoid metabolism is made. The final part (section 4.6) outlines a strategy of future work that is required to follow up the results presented in this thesis.

4.2 Capsaicin biosynthesis as a model system for secondary metabolism

Capsaicin is a typical secondary product as defined by Luckner (1990) and Bell (1981). Its synthesis is specific for some *Capsicum* species and it has no clear physiological function in the plant. Capsaicin is an end product of metabolism (Holden *et al.*, 1987) and changes in the accumulation of this molecule directly reflect

changes in its synthesis. In the plant, it is only synthesised in one highly specialised tissue, the placental cells of the fruit, during a short period of development (Hall *et al.*, 1986). However, it has been shown, that the pathway, which has been under investigation in this laboratory for many years and is relatively well understood, is present and operative in cell cultures of *Capsicum frutescens* (Lindsey and Yeoman, 1984). Indeed, the early steps (from phenylalanine to ferulic acid) of the aromatic branch of the pathway (Fig. 1.4.2) form the core of reactions in the phenylpropanoid metabolism in plant cells.

4.3 The effects of elicitation on the accumulation of soluble phenolic compounds

Elicitation has proved to be a useful tool for the study of secondary metabolism in a number of plant species and the elicitor-stimulated accumulation of secondary metabolites is widely recognised (for a review see Eilert, 1987). The flux of metabolites in biosynthetic pathways of secondary products can be influenced by elicitor treatment, often via the induction of specific enzymes, thus facilitating the molecular analysis of the particular pathway (Hahlbrock and Scheel, 1989). Indeed, several of the molecular probes used to localise pathway activities in whole plant tissues could not have been generated easily from starting material other than elicited cell cultures (for example Cramer *et al.*, 1989).

In this investigation, elicitation was used as a means to study the synthesis and accumulation of phenylalanine derived phenolic compounds in cell cultures of *Capsicum frutescens*.

4.3.1 Free phenolic compounds

Elicitation of *Capsicum* suspension cultures with an aqueous extract of *Gliocladium deliquescens* spores reduced the amount and altered the composition of

phenolics extractable from the culture medium. In addition, a number of unidentified phenolics, present in the medium of non-treated cell cultures, were absent from elicited cultures.

A similar reduction in the amount of major phenolics in the culture medium was detected by Holden *et al.* (1988b) using autoclaved *Gliocladium deliquescens* spores directly to elicit cultures of *Capsicum*. However, the shift in the flow of metabolites towards capsaicin following elicitation, as reported by Holden *et al.* (1988b), was not observed in this study when aqueous extracts of *Gliocladium* spores were used as the source of elicitor, instead, the accumulation of an unidentified "capsaicinoid" was observed. This compound, described tentatively as a "capsaicinoid", displayed a UV spectrum indistinguishable from capsaicin and a R_f during HPLC very close to capsaicin. However, the precise chemical nature of this compound has still to be confirmed. The slightly later R_f of the unidentified "capsaicinoid" suggests that it is slightly less polar than capsaicin. A likely candidate is homocapsaicin which differs from capsaicin only in the length of the side-chain (Fig. 1.4.1).

The fact that only relatively small amounts of radioactivity co-eluted with the induced "capsaicinoid" suggests synthesis from intermediates that are (1) unrelated to phenylalanine or (2) originate from unlabelled pools of phenylalanine related intermediates, perhaps conjugated phenolic compounds (Sukrasno, 1991). Dilution of [^{14}C]phenylalanine by existing pools of unlabelled precursors is also a possibility.

In this study, incorporation of [^{14}C] following elicitation was demonstrated only into some of the intermediates of capsaicin synthesis, cinnamate, coumarate and vanillin. However, a rapid, transient incorporation of radioactivity into other phenylpropanoid pathway intermediates cannot be excluded. Indeed, Hall and Yeoman (1991) have shown that added radiolabel in immobilised, non-elicited *Capsicum* cultures progresses through the phenylpropanoid pathway in 90-180 min and that each of the pools of capsaicin intermediates returns to a basal equilibrium

level after that time. Therefore, the shortest labelling period used for elicited cultures in the present study, 4 h, was probably too long to detect elevated levels of radioactivity in the pools of capsaicin intermediates. Unfortunately, the basal equilibrium levels reported by Hall and Yeoman (1991) are below the detection limit of the autoradiographic method used in this investigation. However, accumulation of the end product of metabolism, capsaicin, was not observed following elicitation. Instead, a transient accumulation of [^{14}C]vanillin (see above) was detected which is consistent with earlier studies of Yeoman *et al.* (1989) who showed that some *Capsicum* cell lines, that do not accumulate capsaicin, produce other pathway intermediates, including vanillin and ferulic acid. The importance of the cell culture line as a variant in the inducibility of secondary metabolism following elicitation has already been pointed out by Eilert (1987). Indeed, the particular *Capsicum* cultures used in this study accumulated measurable amounts of a "vanillin-like" compound (phenolic 7, section 3.7.1.1.2.1) and an unidentified "capsaicinoid" (phenolic 8, section 3.7.1.1.2.1) following elicitor treatment.

Fig. 4.3.1 shows the proposed metabolic routes of vanillin in elicited cultures of *Capsicum frutescens*. Ferulic acid, the supposed precursor of vanillin (Toms and Wood, 1970; Zenk, 1965), was not detected as the free acid, but if free ferulic acid was rapidly converted into various conjugated compounds and into vanillin, no pool of free ferulic acid would be present. Another possibility proposed by Yeoman *et al.* (1980) suggested that vanillin is synthesised from caffeic acid via protocatechuic aldehyde. Again, neither of these precursors was detected following elicitation. Also, vanillin may be formed from *p*-hydroxybenzaldehyde by hydroxylation and methylation at C₃. French *et al.* (1976) showed that *p*-hydroxybenzaldehyde is formed during the conversion of *p*-coumarate to *p*-hydroxybenzoate, although Hagel and Kindl (1975) did not detect *p*-hydroxybenzaldehyde as an intermediate. [^{14}C]coumaric acid was accumulated in controls and elicited cultures markedly above the basal

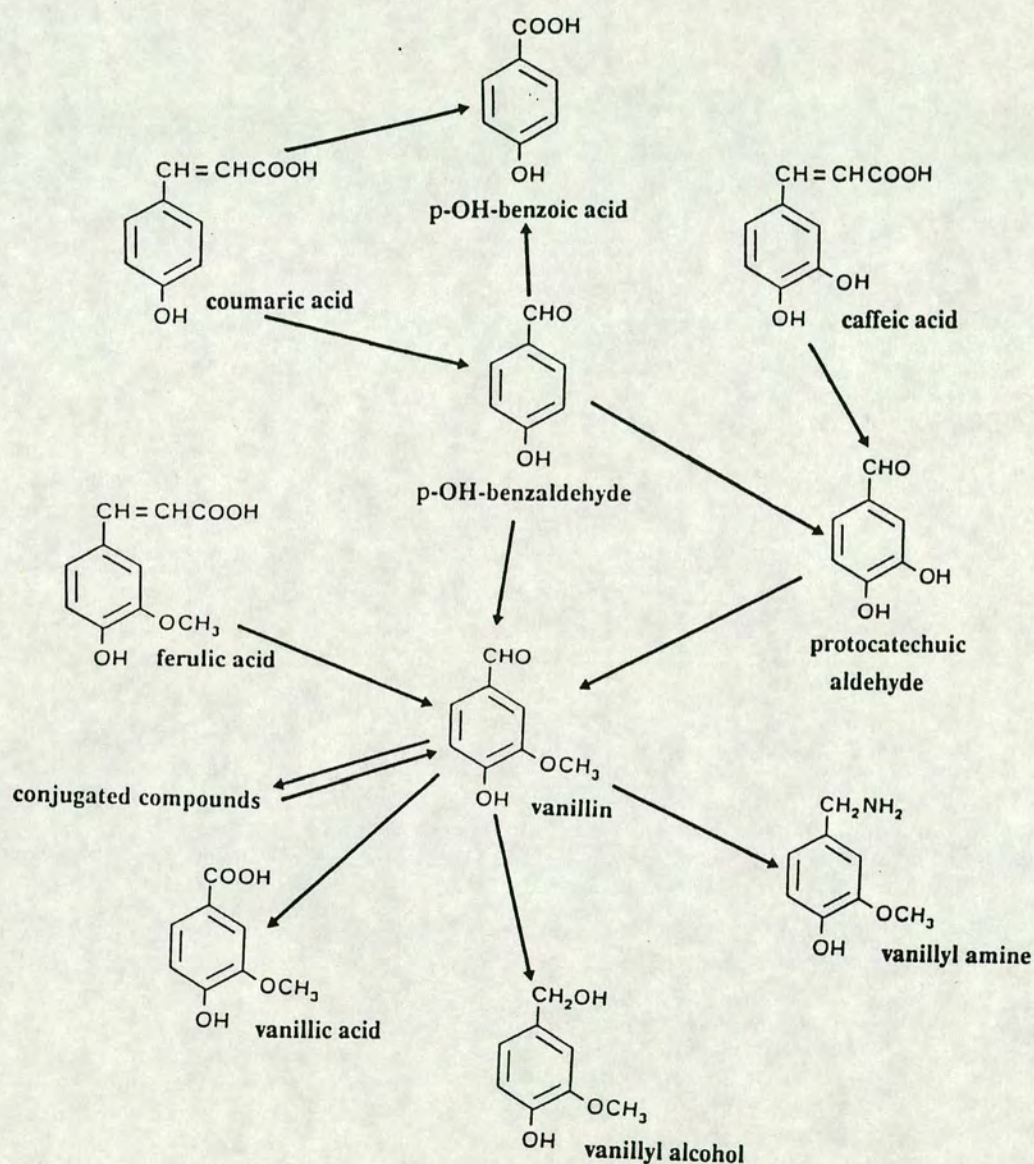


Figure 4.3.1 Proposed metabolic routes of vanillin in elicited cell cultures of *Capsicum frutescens*.

equilibrium levels described by Hall and Yeoman (1991). Therefore, the possibility of a partial conversion of [^{14}C]coumaric acid into [^{14}C]vanillin via labelled *p*-hydroxybenzaldehyde cannot be excluded.

In this study, the accumulation of [^{14}C]vanillin has been shown to be transient, indicating that, unlike capsaicin, vanillin is not an end product of metabolism in this cell culture of *Capsicum frutescens*. In fact, at the height of [^{14}C]vanillin accumulation, a small amount of [^{14}C]vanillyl alcohol was also detectable in the medium, indicating an alternative metabolic route for vanillin. It has already been demonstrated by Bladon (unpublished observation), that feeding vanillin to cultures of *Capsicum frutescens* results in the accumulation of vanillyl alcohol while the bioconversion of vanillin to vanillyl alcohol has also been reported in yeast (De Wulf and Thonart, 1989). However, only small amounts of vanillin were metabolised into vanillyl alcohol during elicitation in this study and most of the vanillin was probably converted into the "capsaicinoid" that accumulated following elicitation. It should also be noted that, although the transiency of the [^{14}C]vanillin accumulation was demonstrated during "short term" elicitation experiments, [^{14}C]vanillin was also detected after elicitation for 72 h. This result suggests that two pools of phenolic intermediates exist (Sukrasno, 1991), one metabolically active and the other a storage pool of conjugated phenolics, so that [^{14}C]feruloyl and other [^{14}C]hydroxycinnamoyl conjugates in the storage pool could serve as precursors of [^{14}C]vanillin at a later stage (72 h), after transfer to the metabolically active pool.

These results indicate that elicitation enhances the flux of metabolites down the phenylpropanoid pathway by making alterations which result in the accumulation of vanillin and a "capsaicinoid", possibly homocapsaicin, instead of capsaicin. It should be noted, that the accumulation of vanillin following elicitation is not a general phenomenon, even elicited cell cultures of *Vanilla planifolia* failed to accumulate

vanillin (Funk and Brodelius, 1990a). It is also known, that elicitation does not always enhance the flux of metabolites down branches of existing pathways, e.g. cultures of *Papaver somniferum* elicited with a range of fungal elicitors accumulated sanguinarine and not the morphinian alkaloids as expected (Eilert *et al.*, 1985). In this investigation, the increased flux of metabolites following elicitation did not result in increased levels of free phenolic compounds in general. Indeed, the total amount of free phenolics was reduced, with the exception of the specific induction of vanillin and homocapsaicin accumulation.

4.3.2 Conjugated phenolic compounds

Previous research on phenylpropanoid metabolism in *Capsicum frutescens* has largely concentrated on the conversion of free phenolics, the supposed intermediates of capsaicin (Hall and Yeoman, 1991; Hall *et al.*, 1986;1987; Holden *et al.*, 1987). However, Sukrasno (1991) pointed out the importance of various groups of conjugated phenolic compounds as possible active intermediates of capsaicin synthesis.

In this study, a strong, transient increase in water soluble conjugated phenolic compounds, possibly phenolic sugar conjugates, was observed following elicitation. This induction of conjugated phenolics followed the same pattern of accumulation observed for [¹⁴C]vanillin. Earlier, Sukrasno (1991) demonstrated that sugar conjugates of ferulic acid (sugar esters and O-glycosides) accumulate in *Capsicum* cells during the culture cycle. Although, free ferulic acid was not detected in the cell cultures used in this investigation, a proportion of the phenolic conjugates that accumulated during elicitation are likely to be conjugates of ferulic acid which could be involved in the synthesis of vanillin via feruloyl conjugates.

It is perhaps significant, that the major conjugated phenolic accumulated following elicitation was identical to the compound tentatively identified as 3,4-dimethoxycinnamoyl glycoside by Sukrasno (1991). Funk and Brodelius (1990c)

demonstrated the synthesis of vanillic acid from 3,4-dimethoxycinnamic acid in cultures of *Vanilla planifolia* and subsequently, questioned the role of ferulic acid as a precursor of vanillic acid (Funk and Brodelius, 1990b). Indeed, 3,4-dimethoxycinnamoyl glycoside may act as a precursor for vanillin, but its major role, along with the bulk of the hydroxycinnamoyl conjugates that accumulate transiently following elicitor treatment, is in the synthesis of cell wall components (Sukrasno, 1991), which forms part of a defence mechanism against fungal attack. The synthesis of ligneous material, as well as phytoalexins, following fungal elicitation of plant cell cultures has been reported for a number of plant systems (see Bolwell, 1988). Hahlbrock and Scheel (1989) reported rapid incorporation of phenolic compounds into the cell walls of parsley cells following elicitation but, this was due to the esterification of hydroxycinnamic acids to the cell wall components and not due to an increase in lignification. Hahlbrock and Scheel (1989) concluded that the impregnation of the cell wall with these esters in parsley is an early stage of the response against the pathogen. In this context, the synthesis of vanillin and any "capsaicinoid" might only be a side effect triggered by the sudden availability of intermediates common to cell wall synthesis and other pathways, like capsaicin biosynthesis. There are many branch points along the phenylpropanoid pathway where intermediates may be channelled into a variety of products. It has been proposed, that the key regulatory enzyme for capsaicin synthesis, capsaicin synthase, is located at the end of the pathway (Holland, 1989). However, the first enzymes of phenylpropanoid metabolism are equally important because they make the precursors available for the metabolic sequence leading to capsaicin. Holden *et al.* (1988a,b) have shown that increased incorporation of [^{14}C]precursors into capsaicin, in elicited cultures of *Capsicum frutescens*, was correlated with increased PAL activity which was not observed during other treatments that increased [^{14}C] incorporation into capsaicin, including precursor feeding (Yeoman *et al.*, 1980), cell immobilisation (Lindsey,

1986), and nutrient limitation (Lindsey, 1985). Therefore, attention was focussed on the regulation of PAL induction during elicitation and its effects on phenylpropanoid metabolism.

4.4 The effects of elicitation on PAL

4.4.1 PAL activity

The enhanced availability of phenylpropanoid precursors in elicited cell cultures, which is necessary to support the observed accumulation of vanillin, capsaicinoids, and conjugated phenolic compounds, clearly points to changes in the activity of PAL.

Holland (1989) has shown that the activity of this enzyme in *Capsicum* cell cultures increased transiently following subculture. After this initial induction, the so-called transfer or dilution effect, that has been demonstrated in a number of cell culture systems (Cvikrová *et al.*, 1991; Ozeki *et al.*, 1990; Ozeki and Komamine, 1985; Jones, 1984; Hahlbrock, 1976; Hahlbrock and Schröder, 1975; Hahlbrock and Wellmann, 1973), PAL activity is known to stay at a low basal level throughout the growth cycle of cultures that are maintained in the presence of 2,4-D (e.g. Ozeki *et al.*, 1989). However, culture age can have a great influence on the response of cells to elicitor treatment (Brodelius *et al.*, 1989; Eilert, 1987). In this investigation, the highest inducibility of PAL activity following elicitation was observed in the early stationary phase although, the highest amount of extractable PAL activity was detected in the early part of the growth cycle. This is consistent with the results of Kurosaki *et al.* (1986) who also reported the highest inducibility of PAL activity, following elicitation of carrot cell cultures, in the early stationary phase and the highest basal PAL activity in the early logarithmic phase of culture growth.

In this study, PAL activity was induced rapidly following the addition of elicitor to cultures of *Capsicum frutescens*. There was a tendentious increase in PAL activity 20 min after the introduction of the fungal elicitor followed by a significant increase in

PAL activity after 40 min. These early events of the induction of PAL activity have, so far, not been investigated in any detail but deserve careful consideration. The observed induction of PAL activity in pepper cultures was transient, reaching a maximum after *ca.* 8 h, and the overall pattern was similar to that reported for elicited cell cultures of parsley (Kombrink and Hahlbrock, 1986) and bean (Bolwell *et al.*, 1985).

Three different mechanisms, or combinations of the three, which may be responsible for the observed induction of PAL activity are considered. (1) The activation of enzyme already present. (2) Changes in the turnover of the enzyme, i.e. transient reduction of the rate of the removal (degradation and inactivation) of the active enzyme. (3) *De novo* synthesis of the enzyme. Since PAL is known to be newly synthesised upon elicitation in other plant systems (see Dixon *et al.*, 1986), the *de novo* synthesis of PAL protein in pepper cultures was perhaps inevitable.

4.4.2 PAL protein

4.4.2.1 Characterisation of PAL protein sub-units

PAL is reported to be a tetrameric enzyme consisting of identical sub-units (Jones, 1984). Reported values for the molecular size of the native enzyme sub-units range from 83 kD to 55 kD (Bolwell and Rodgers, 1991; Bolwell *et al.*, 1985; Gupta and Acton, 1979). A list of the molecular sizes of PAL protein sub-units is contained in Table 4.4.1. In this study, *Capsicum* and *Petroselinum* PAL protein sub-units were identified by immunoblotting using anti-(parsley PAL) serum. Their molecular sizes were identical and estimated to be *ca.* 77 kD using SDS-PAGE. This is consistent with the M_r reported previously for parsley (Lawton *et al.*, 1983a), bean (Dixon *et al.*, 1986), and sweet potato (Tanaka *et al.*, 1989).

Different isoforms of *Capsicum* PAL protein sub-units, similar to those reported from other plant sources (Hahlbrock and Scheel, 1989; Dixon *et al.*, 1986), are

Table 4.4.1 A list of the molecular sizes of PAL protein sub-units reported for various plant species.

size [kD]	species	comment	Reference
86	<i>Bambusa oldhami</i>	constitutive	Chen <i>et al.</i> (1988)
77 70	<i>Capsicum frutescens</i> <i>Capsicum frutescens</i>	elicitation degradation	this study this study
70	<i>Cuscuta chinensis</i>	lignification	Nagajah <i>et al.</i> (1977)
79 77	<i>Daucus carota</i> <i>Daucus carota</i>	UV, elicitation isoform or degradation	Gleitz <i>et al.</i> (1991) Gleitz <i>et al.</i> (1991)
72	<i>Fragaria ananassa</i>	fruit ripening	Given <i>et al.</i> (1988)
78 72	<i>Glycine max</i> <i>Glycine max</i>	elicitation <i>in vitro</i> translat.	Ebel <i>et al.</i> (1984) Ebel <i>et al.</i> (1984)
58/68	<i>Helianthus annuus</i>	constitutive 2 x 2 sub-units	Jorin <i>et al.</i> (1988)
77	<i>Ipomoea batatas</i>	wounding	Tanaka <i>et al.</i> (1989)
74	<i>Lycopersicon esculentum</i>	elicitation	Bernards & Ellis (1991)
79	<i>Medicago sativa</i>	elicitation	Gowri <i>et al.</i> (1991)
76	<i>Oryza sativa</i>	UV irradiation	Minami <i>et al.</i> (1989)
83 78 77 73 70/53	<i>Phaseolus vulgaris</i> <i>Phaseolus vulgaris</i> <i>Phaseolus vulgaris</i> <i>Phaseolus vulgaris</i> <i>Phaseolus vulgaris</i>	constitutive calculated from cDNA elicitation <i>in vitro</i> translat. degradation	Bolwell & Rodgers (1991) Cramer <i>et al.</i> (1989) Bolwell <i>et al.</i> (1985) Lawton <i>et al.</i> (1983b) Bolwell <i>et al.</i> (1986a)

Table 4.4.1 continued

size [kD]	species	comment	Reference
84	<i>Petroselinum hortense</i>	UV irradiation	Hahlbrock & Schröder (1975)
83	<i>Petroselinum hortense</i>	UV irradiation	Zimmermann & Hahlbrock (1975)
78	<i>Petroselinum crispum</i>	calculated from cDNA	Lois <i>et al.</i> (1989)
77	<i>Petroselinum hortense</i>	elicitation	Lawton <i>et al.</i> (1983a)
77	<i>Petroselinum hortense</i>	constit. (leaves)	this study
76	<i>Petroselinum hortense</i>	UV, elicitation	Kuhn <i>et al.</i> (1984)

possible but, were not detected using the separation method employed in this investigation.

In elicited and non-elicited cultures of *Capsicum frutescens* a 70 kD polypeptide was recognised by anti-(parsley PAL) serum that was not detected in parsley extracts. At first, it was not clear whether this polypeptide was related to PAL, however, the antibody-antigen complex formed with the 70 kD protein was stable under stringent washing conditions thus, indicating structural similarities to PAL sub-units. Indeed, the native PAL subunit of M_r 77 kD in French bean is reported to be inherently unstable and to yield partial degradation products of M_r 70 kD and, to a lesser extent, of M_r 53 kD (Bolwell *et al.*, 1986a). In this study, the 70 kD polypeptide was present in similar quantities in controls and elicited cultures and the difference in PAL activity measured between elicited and non-elicited cultures resulted from the specific induction of the synthesis of 77 kD PAL protein sub-units. However, the synthesis of PAL sub-units at a low level was detected in control cultures using *in vivo* labelling experiments. The amount of enzyme synthesised in untreated *Capsicum* cultures, in conjunction with the partially degraded sub-units of M_r 70 kD, may account for the

low basal levels of PAL activity measured. Indeed, Bolwell *et al.* (1986a) suggested that the 70 kD and 53 kD components detected in *Phaseolus* preparations contribute to an active enzyme protein although, such partially degraded preparations exhibited a lower specific activity than the intact enzyme. The inability to recognise small amounts of the 77 kD sub-units on immunoblots of extracts from non-elicited *Capsicum* cultures by PAL antibodies might be a result of a much lower specificity of the anti-(parsley PAL) serum for *Capsicum* PAL protein. Indeed, hybridisation to the small amounts of 77 kD sub-units present in control cultures was possibly removed during the stringent washings necessary to reduce nonspecific hybridisation. The lower specificity of the antiserum and consequently, a partial removal of bound antibodies during the washing procedures may also explain the observed similarity in strength of the PAL signals obtained from parsley extracts and extracts from elicited *Capsicum* cultures, despite the higher PAL specific activity of the latter (10x). A less likely explanation for the similarity in signal strength would be that the differences in specific PAL activity estimated for the crude enzyme extracts from *Petroselinum* and *Capsicum* are not a result of different amounts of enzyme present but of completely different kinetic properties of PAL from parsley and chilli pepper.

Immunoprecipitation of *Capsicum* PAL protein with anti-(parsley PAL) serum yielded a number of polypeptides with approximate molecular weights of 81-57 kD. From this it was concluded, that the antiserum was not monospecific for the enzyme from *Capsicum*, which is consistent with the reported lack of monospecificity of the anti-(parsley PAL) serum for bean PAL (Bolwell *et al.*, 1985). However, in the context of recently published results on the differential expression of antigenic multiple M_r forms of bean PAL (Bolwell and Rodgers, 1991) a second explanation has to be considered for the observed co-precipitations in *Capsicum* extracts. Bolwell and Rodgers (1991) showed that in French bean, apart from the well characterised stress inducible M_r 77 kD forms of PAL, a constitutive form of PAL with sub-units of

M_r 83 kD is recognised at high concentrations of antiserum to antigen, which is possibly involved in low-level accumulation of phenolics. An M_r 83 kD form of PAL has also been reported for extracts from bean leaf (Da Cunha, 1988) and a number of the earlier purifications of PAL from plant tissue had a M_r of 83 kD rather than 77 kD (Table 4.4.1). The immunoprecipitations in this study were performed at relatively high levels of antiserum and the low levels of phenolic compounds accumulated in *Capsicum* cultures at stationary phase could involve a constitutive form of PAL. The estimated sizes of the immunoprecipitated polypeptides, 81-57 kD, are close to the size range for PAL related proteins in French bean (Bolwell and Rodgers, 1991; Bolwell *et al.*, 1986a), i.e. 83 kD for a constitutive form of PAL, 77 kD for stress-induced forms of PAL, and 70 kD and 53 kD for partial degradation products of stress-induced forms of PAL. Thus, the polypeptides immunoprecipitated by anti-(parsley PAL) serum in *Capsicum* extracts could indeed represent PAL related proteins. Furthermore, the expression of a constitutive form of PAL, different from the enzyme induced by fungal elicitor, has to be considered.

4.4.2.2 Synthesis of "PAL" protein following elicitation

Results of Western Blots presented in this thesis demonstrated that elicitor treatment of *Capsicum* cell cultures induced the accumulation of 77 kD "PAL" protein sub-units, which is consistent with the results described for elicited cell cultures of parsley (Hahlbrock *et al.*, 1981) and bean (Dixon *et al.*, 1986). Pulse labelling experiments showed that the "PAL" sub-units were actively synthesised following elicitor treatment and labelled "PAL" sub-units accumulated transiently, with a maximum 4 h after elicitor induction. It is perhaps significant, that the proportion of radiolabelled "PAL", relative to the total amount of radiolabelled translation products, was highest after 8 h elicitation thus, facilitating the highest specific PAL activity although, the absolute amount of radiolabelled "PAL" sub-units peaked at 4 h together

with the total amount of radiolabelled proteins. In contrast to these results, Dixon *et al.* (1986) showed, using elicitor-treated bean cell cultures, that maximum levels of immunodetectable PAL protein were induced after 8-10 h.

Active synthesis of "PAL" protein in *Capsicum* cultures was induced almost immediately after the addition of the elicitor, a similar response to that observed in elicited cultures of parsley (Hahlbrock *et al.*, 1981) and bean (Cramer *et al.*, 1985). A maximum rate of PAL synthesis in elicited cultures of bean and parsley is reported to occur 3 h after elicitation (Cramer *et al.*, 1985; Hahlbrock *et al.*, 1981). With the methods used in this study an elevated, approximately constant, rate of "PAL" synthesis was detected between 40 min and 4 h which decreased thereafter.

It should be noted, that, apart from "PAL" sub-units, the synthesis of a 32 kD polypeptide was transiently induced following elicitation with a maximum after 8 h. The identity of this 32 kD protein is unknown but, its M_r is identical to that of unprocessed chitinase precursor (Gomez Lim *et al.*, 1987). Indeed, chitinase (EC 3.2.1.14) activity is known to be induced during elicitation in a number of plant systems, including cucumber (Metraux and Boller, 1986), pea (Mauch *et al.*, 1984), carrot (Kurosaki *et al.*, 1986), and parsley (Chappell *et al.*, 1984). The observed induction of the 32 kD protein, at a later stage during the elicitor treatment of *Capsicum* cells, is consistent with the reported lag of chitinase induction in elicited parsley cells (Chappell *et al.*, 1984) although, in elicited carrot cells chitinase activity increased first, followed by an increase in PAL activity (Kurosaki *et al.*, 1986).

Chitinase has been found in a number of plants but the substrate has not been detected so far (Zhu and Lamb, 1991; Boller *et al.*, 1983; Pegg and Young, 1981; Molano *et al.*, 1979; Wargo, 1975). It is likely, that the substrate must come from another organism and fungi are good candidates, as chitin is the main constituent of fungal cell walls (Bartnicki-Garcia, 1968). Abeles *et al.* (1970) suggested that

chitinase functions as a defence mechanism in plants. The role of chitinase in this defence mechanism possibly lies not only in the direct attack on the invading pathogen (Hughes and Dickerson, 1991) but chitinase activity may also release more elicitor molecules from the fungal cell walls (Kurosaki *et al.*, 1988) which, in turn, renew the elicitation signal as demonstrated by Kurosaki *et al.* (1986) using carrot cell cultures.

4.4.3 PAL mRNA

In this study, increased steady state levels of PAL mRNA, estimated by Northern Blot experiments, correlated with the observed transient increase in "PAL" protein synthesis thus, indicating that "PAL" *de novo* synthesis, following elicitation of *Capsicum* cells, is regulated at the level of transcription. The rapid increase of PAL mRNA from very low basal levels also suggests that elicitation stimulates mRNA synthesis. Indeed, it has been shown for parsley and bean cell cultures, that increased levels of PAL mRNA following elicitor treatment resulted from the induction of PAL gene transcription (Lois *et al.*, 1989; Lawton and Lamb, 1987).

The close correspondence between the kinetics of the accumulation of PAL mRNA and "PAL" protein sub-units indicates that the transient increase in PAL mRNA, following elicitation of cell cultures of *Capsicum frutescens*, is the major factor governing the rate of enzyme synthesis throughout the phase of rapid increase in enzyme activity. Edwards *et al.* (1985) suggested that post-transcriptional control mechanisms may become important in later stages of the response, especially in relation to the subsequent rapid decay in hybridisable PAL mRNA.

Capsicum cells responded very fast to elicitor treatment in this study and PAL mRNA levels increased strongly from very low basal levels after only 20 min. Indeed, Lawton and Lamb (1987) demonstrated that PAL gene transcription in bean cells was activated within 5 min of the addition of the elicitor. The rapid activation of the transcription of genes encoding so-called pathogenesis-related proteins, some of

which were identified to be chitinases and glucanases (Kombrink *et al.*, 1988; Kauffmann *et al.*, 1987; Legrand *et al.*, 1987), in elicitor-treated parsley cells has been shown by Somssich *et al.* (1986). The rapidity of the induction of bean PAL genes (Lawton and Lamb, 1987), and possibly *Capsicum* PAL genes (this study), by the elicitor implies that the signal transduction system is in place prior to the elicitor treatment and that there are few intervening steps between the elicitor binding to a putative plant cell receptor (Cheong and Hahn, 1991; Schmidt and Ebel, 1987; Darvill and Albersheim, 1984; Dixon *et al.*, 1983; Sequeira, 1983) and the specific transcriptional activation of these genes. A specific transcriptional activation by the elicitor in this study is considered because the total amount of extractable RNA remained practically unchanged and indeed, Cramer *et al.* (1985) demonstrated that, although elicitation stimulated the synthesis of a large number of mRNA species, the synthesis of some mRNA species was unaffected by the elicitor and the synthesis of several mRNA species was decreased following elicitor treatment.

The first measurable reaction to elicitor-binding is possibly the immediate increase of cytoplasmic calcium, demonstrated by Knight *et al.* (1991) using the same *Gliocladium deliquescens* preparation used in this investigation to elicit transgenic *Nicotiana* seedlings, and the elicitor response in soybean (Stab and Ebel, 1987) and parsley cells also requires the presence of calcium ions (Hahlbrock, personal communication). Calcium may activate protein kinases resulting in a rapid, transient, and sequential phosphorylation of a number of proteins (Felix *et al.*, 1991b; Dietrich *et al.*, 1990). The short cascade of signal transduction also seems to involve *cis*-acting elements that were detected in the promoter regions of PAL genes from parsley and bean (Lois *et al.*, 1989; Cramer *et al.*, 1989) and in genes encoding pathogenesis-related proteins (Meier *et al.*, 1991).

The transfer-effect on PAL activity in cell cultures of *Capsicum frutescens* described by Holland (1989) possibly involves similar mechanisms as the elicitation

Table 4.4.2 A list of the molecular sizes and inducibility of PAL mRNAs reported for various plant species.

size [kb]	species	comment	Reference
2.6	<i>Capsicum frutescens</i>	dilution	this study
2.6	<i>Capsicum frutescens</i>	elicitation	this study
2.4	<i>Daucus carota</i>	dilution	Ozeki <i>et al.</i> (1990)
2.6	<i>Glycine max</i>	elicitation	Habereder <i>et al.</i> (1989)
2.6	<i>Glycine max</i>	elicitation	Ebel <i>et al.</i> (1984)
2.4	<i>Ipomoea batatas</i>	wounding	Tanaka <i>et al.</i> (1989)
2.8	<i>Lycopersicon esculentum</i>	elicitation	Bernards & Ellis (1991)
2.4	<i>Medicago sativa</i>	elicitation	Gowri <i>et al.</i> (1991)
2.5	<i>Oryza sativa</i>	UV irradiation	Minami <i>et al.</i> (1989)
2.5	<i>Phaseolus vulgaris</i>	elicitation	Lawton <i>et al.</i> (1983b)
2.5	<i>Phaseolus vulgaris</i>	elicitation	Edwards <i>et al.</i> (1985)
2.5	<i>Phaseolus vulgaris</i>	cDNA clone	Cramer <i>et al.</i> (1989)
3.3	<i>Petroselinum hortense</i>	UV irradiation	Ragg & Hahlbrock (1980)
2.7	<i>Petroselinum hortense</i>	UV irradiation	Kuhn <i>et al.</i> (1984)
2.7	<i>Petroselinum hortense</i>	elicitation	Kuhn <i>et al.</i> (1984)
2.4	<i>Petroselinum crispum</i>	cDNA clone	Lois <i>et al.</i> (1989)
2.3	<i>Zinnia elegans</i>	differentiation	Lin & Northcote (1990)

of *Capsicum* cells since elevated levels of PAL mRNA were measured in this study following dilution of cell cultures. This is consistent with the results of Ozeki *et al.* (1990) who showed that the dilution-effect on PAL in carrot cultures is a result of induced PAL gene transcription.

The inducibility of *Capsicum* PAL mRNA by stress and its molecular size are very similar to PAL mRNA from other plant sources (Table 4.4.2), supporting the similarities detected for PAL protein (Table 4.4.1).

4.4.4 Possible mode of regulation of PAL

The strong similarities in the properties and induction pattern of *Capsicum* PAL and PAL from other plant sources indicate that PAL induction by elicitor treatment of plant cell cultures may be achieved by common, or very similar, regulatory mechanisms. In this section, the possible mechanisms involved in the regulation of PAL induction following elicitation of cell cultures of *Capsicum frutescens* will be summarised, considering the evidence obtained in this investigation supplemented with results reported for other plant species.

The first step in the chain of reactions that follows the addition of fungal elicitor to plant cell cultures is likely to be the binding of the elicitor to plant receptor molecules on the cell surface (Schmidt and Ebel, 1987) and high-affinity binding sites for elicitor molecules have recently been identified as proteins or glycoproteins (Cheong and Hahn, 1991).

The binding of the elicitor to receptor molecules (see Fig. 1.3.1) triggers an immediate and transient increase in cytoplasmic calcium (Knight *et al.*, 1991) and indeed, Ca^{2+} was identified as mediator of intracellular transmission of elicitor response in soybean (Stab and Ebel, 1987).

The increase of cytoplasmic calcium results in a rapid (1 min after the addition of the elicitor), transient and sequential phosphorylation of a number of proteins, which

correlates with the activation of defence genes (Dietrich *et al.*, 1990). The short cascade of signal transduction (Lawton and Lamb, 1987) also seems to involve *cis*-acting elements (Lois *et al.*, 1989; Cramer *et al.*, 1989) resulting in the specific induction of PAL gene transcription within 5 min of the addition of the elicitor (Lawton and Lamb, 1987). Thus induced PAL gene transcription results in the rapid increase in steady state levels of PAL mRNA in *Capsicum* cells, which is likely to be the major factor governing the rate of enzyme synthesis throughout the phase of rapid increase in enzyme activity.

Induced chitinase activity may enhance the elicitor response during pathogen attacks via the release of more elicitor molecules from microbial cell walls (Kurosaki *et al.*, 1986). Additionally, oligosaccharides released from pathogen cell walls by lytic enzymes may induce defence responses in other plant cells and, in the whole plant, may lead to induced systemic resistance and lesion limitation (Lamb *et al.*, 1989). Furthermore, endogenous elicitors released from the host cell wall (Dixon *et al.*, 1989) by mechanical damage prior to infection may sensitise the plant to low levels of exogenous elicitor (Lamb *et al.*, 1989).

Post-transcriptional control mechanisms may become important in later stages of the response (Edwards *et al.*, 1985) and include the *in vivo* degradation of native enzyme sub-units (77 kD) to smaller polypeptides (Bolwell *et al.*, 1986a). Cinnamic acid, the product of PAL activity, may down-regulate PAL transcription and may induce the removal of active PAL via the synthesis of an irreversible inactivator of the enzyme (Gowri *et al.*, 1991). Control mechanisms, including degradation, inactivation, and down-turn of transcription, facilitate the transiency of the induction of specific PAL activity following elicitation.

4.5 The effects of PAL induction on phenylpropanoid metabolism

Treatments of cell cultures of *Capsicum frutescens* that have been shown to increase the incorporation of labelled precursors into capsaicin, including cell immobilisation (Lindsey, 1986), nutrient limitation (Lindsey, 1985) and precursor feeding (Yeoman *et al.*, 1980), did not involve an increase in PAL activity. However, Holden *et al.* (1988a,b) have shown that an increased incorporation of labelled precursors into capsaicin, following elicitation of *Capsicum* cells, was correlated with increased PAL activity. The effects of elicitation on PAL in cell cultures of *Capsicum frutescens* were analysed in detail in this study and it was demonstrated that PAL activity increased transiently following the addition of fungal elicitor, possibly due to the transient induction of PAL gene transcription. The observed PAL induction however, did not result in the increased incorporation of labelled precursors into capsaicin in the cell cultures used in this study although, small amounts of "capsaicinoids" were detected following elicitation.

It is perhaps significant, that water soluble conjugated phenolics accumulated transiently, following closely the elicitor-mediated induction pattern of PAL activity. These phenolic compounds are believed to be involved in the synthesis of cell wall components (Sukrasno, 1991) and a mechanism of impregnating cell walls with phenolic esters against pathogen attack, as observed in elicited parsley cells (Hahlbrock and Scheel, 1989), has to be considered. In fact, the synthesis of these phenolics may be the main objective of the elicitor-induced PAL activity since PAL in *Capsicum* is not involved in the synthesis of phytoalexins because the phytoalexins of *Capsicum*, and most other members of the *Solanaceae* (Ebel, 1986), are sesquiterpenes (Brooks *et al.*, 1986). Although, PAL in *Capsicum* is the first enzyme in the biosynthetic pathway leading to capsaicin, elicitation is known to - often unexpectedly - redirect the flow of metabolites into different branches of existing pathways to produce defence-related compounds (Eilert *et al.*, 1985; Furze *et al.*,

1991). The flow of metabolites may be directed via the regulation of the transient expression of enzymes along the biosynthetic pathways as demonstrated for a number of enzymes (Hahlbrock and Scheel, 1989) including chalcone synthase, the gene expression of which has been shown to be (a) regulated by phenylpropanoid intermediates (Loake *et al.*, 1991), (b) induced directly by UV light (Gleitz *et al.*, 1991) and elicitors (Loake *et al.*, 1991; Haberer *et al.*, 1989) and (c) suppressed directly by elicitor (Gleitz *et al.*, 1991). In this study, elicitor treatment increased the flux of metabolites down the phenylpropanoid pathway resulting in the transient accumulation of vanillin in *Capsicum* cells but, only small amounts of the [¹⁴C]vanillin synthesised were incorporated into "capsaicinoids". Thus, possible regulatory steps may include the depression of the postulated phenylalanine:vanillin aminotransferase (Kopp *et al.*, 1983) by the elicitor and the induction of enzymes that catalyse the synthesis of vanillin conjugates.

Fig. 4.4.1 is a simplified model of the flux of metabolites in and out of the capsaicin biosynthetic pathway after the addition of the fungal elicitor.

It has been shown for a number of plant species, including parsley (Lois *et al.*, 1989) and French bean (Cramer *et al.*, 1989), that PAL is encoded by a small multigene family and indeed, elicitor-treated bean cells exhibit a differential induction of low- K_M isoforms (Bolwell *et al.*, 1985) that are different from constitutive bean PAL (Bolwell and Rodgers, 1991), thereby exerting a metabolic priority of phenylpropanoid synthesis in the cellular economy of phenylalanine under conditions of stress (Lamb *et al.*, 1989). Furthermore, a diverse polymorphism of PAL in carrot cells has been reported by Ozeki *et al.* (1990) who showed, that the enzyme induced rapidly and transiently by stress is different from the PAL induced slowly during differentiation and anthocyanin synthesis. Also, studies on the differential expression of PAL during soybean nodule development have shown that not symbiosis-specific

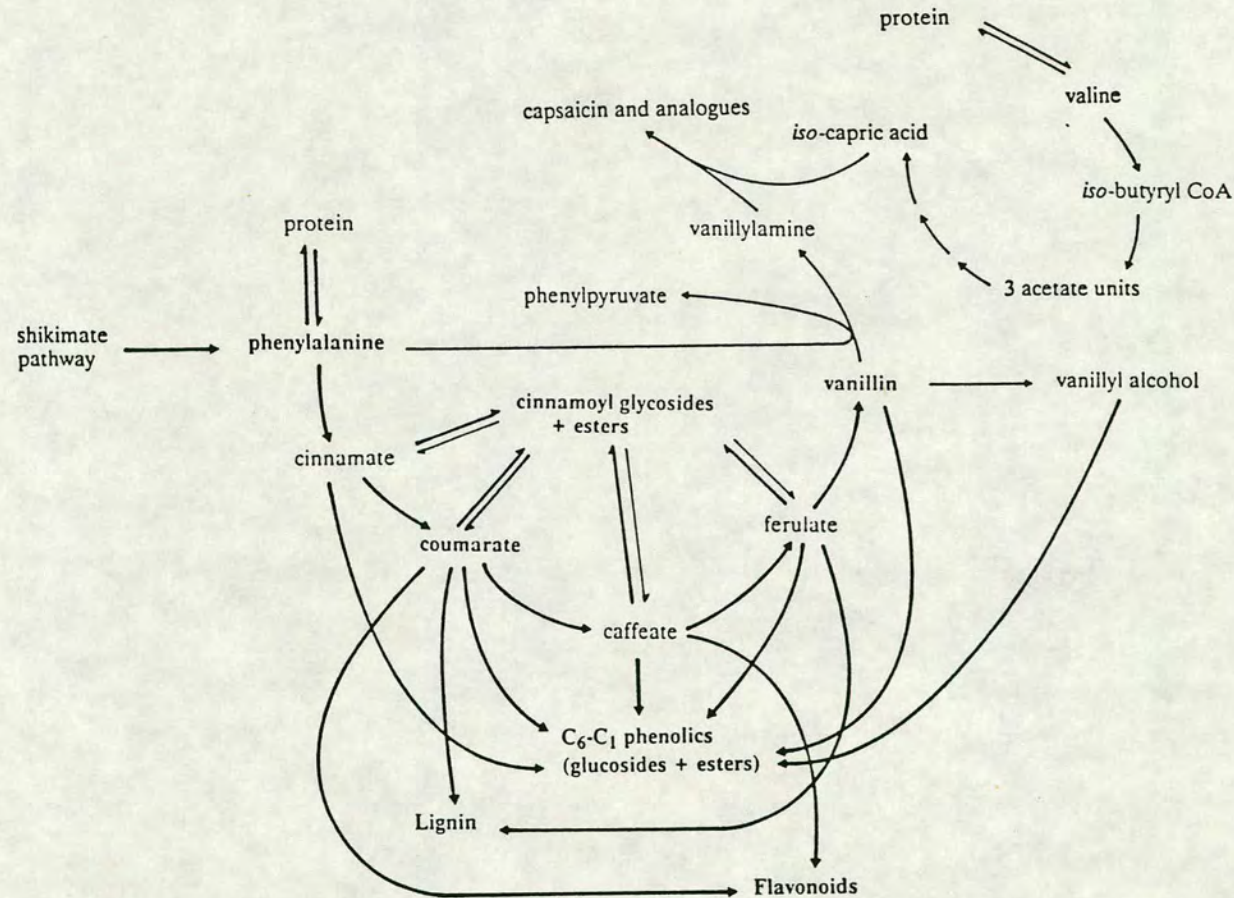


Figure 4.4.1 Proposed metabolic balance within the capsaicin biosynthetic pathway in *Capsicum* cells after the addition of the fungal elicitor. Bold arrows represent enhanced flux of metabolites, names of intermediates in bold print indicate accumulation.

PAL genes but different subsets of PAL genes are induced by stress or pathogen interaction (Estabrook and Sengupta-Gopalan, 1991). The selective expression of PAL genes encoding functional variants seems to be governed by a complex set of regulatory networks for developmental and environmental control of phenylpropanoid biosynthesis (Liang *et al.*, 1989) which might involve coordinated regulation for the biosynthesis of primary and secondary compounds (Dyer *et al.*, 1989; McCue and Conn, 1990; 1989). Indeed, the expression of a PAL gene obtained from elicitor-treated bean cells in transgenic tobacco leads to the suppression of endogenous PAL, abnormal plant development, and down-regulation of phenylpropanoid biosynthesis (Elkind *et al.*, 1990).

The existence of multiple forms of PAL in *Capsicum* with differential expression during capsaicin synthesis, dilution and elicitor treatment has also to be considered. In fact, the induction of PAL in elicitor-treated *Capsicum* cells has to be seen in the context of biosynthetic machinery directed towards defence against pathogen attack, resulting in a decrease of phenolic compounds released into the culture medium, phytoalexin synthesis (Brooks *et al.*, 1986), chitinase induction and possible impregnation of cell walls with phenolic esters (Hahlbrock and Scheel, 1989). PAL induction might be required to provide precursors for the latter which means that these precursors are prevented from entering the biosynthetic pathway leading to capsaicin. This might be achieved by differential expression of inducible forms of PAL, possibly with kinetic properties different from constitutive PAL and perhaps targetted to different cell compartments, concomitant with a possible suppression of constitutive forms of PAL involved in developmentally regulated capsaicin biosynthesis.

4.6 Future work

This investigation has contributed to the elucidation of the regulation of PAL induction following elicitation of cell cultures of *Capsicum frutescens* and described its effects on certain aspects of phenylpropanoid metabolism, but there are many questions which still remain unanswered. Further molecular characterisation of PAL is required to elucidate the complexity of the regulation of PAL expression indicated by the results of this study.

The long term aim of the proposed future work is to genetically manipulate PAL in capsaicin synthesis so that its amount of expression is increased and more metabolites are channelled into capsaicin synthesis. Therefore, the isoform of PAL that is involved in capsaicin biosynthesis has to be identified and thus, two major aspects of the regulation of the enzyme need to be investigated: 1) The spatial distribution of PAL expression and 2) the differential expression of functional isoforms.

4.6.1 Spatial distribution of PAL expression

- a) It is crucial for further studies on the regulation of PAL in *Capsicum* to raise antibodies against the purified enzyme protein from *Capsicum* because antibodies against PAL from other plant sources proved unreliable. Elicited cell cultures of *Capsicum frutescens* would be potentially a good source material for PAL purification because of the high level of PAL expression.
- b) The role of compartmentation at a cellular level should be investigated using immunolocalisation experiments with *Capsicum* PAL antibodies. Comparative studies using epidermal cells of placental tissue from *Capsicum* fruit before, during and after the onset of capsaicin synthesis and from fruit inoculated with a fungal elicitor would show if the spatial distribution of PAL expression at a

cellular level is involved in channelling metabolites into capsaicin synthesis. Comparative studies on the cellular compartmentation of PAL expression using cell cultures subjected to dilution, UV irradiation and elicitor treatment would be necessary to study differences in PAL induction.

- c) Immunolocalisation experiments could also be used to study tissue specific expression of PAL in fruit, leaf, stem and root of *Capsicum frutescens* plants which should be compared to differences in phenylpropanoid metabolism.

4.6.2 Differential expression of functional isoforms of PAL

- a) The first step in this line of investigation is to determine if PAL in *Capsicum* is encoded by a multigene family. This could be achieved by Southern Blot analysis of genomic DNA probed with labelled PAL cDNA.
- b) The preparation of a cDNA library containing the clones of different subsets of PAL genes would be necessary to provide probes for the study of differential expression.
- c) The study of differential transcription of functional isoforms could be achieved by differential inhibition of synthesis *in vitro* with gene-specific antisense transcripts followed by comparative 2-D gel electrophoresis of polypeptides produced. Again, comparative studies should include epidermal cells of placental tissue from *Capsicum* fruit before, during and after the onset of capsaicin synthesis and from elicited fruit, as well as cell cultures subjected to dilution, UV irradiation and elicitor treatment.

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CHANGES OF PAL ACTIVITY AND mRNA LEVELS IN CHILLI PEPPER CULTURES FOLLOWING ELICITATION AND DILUTION

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Suspension cultures of Capsicum frutescens were initiated from stem segments of pepper plants and maintained on a Schenk & Hildebrandt medium. Elicitation with aqueous preparations of Gliocladium deliquescens spores containing 900µg carbohydrates triggered an up to 10-fold increase in phenylalanine ammonia-lyase (PAL) activity. PAL activity reached a maximum ca. 8 hours after addition of the fungal elicitor and was still above the level of controls after 16 hours. A similar transient effect was observed upon dilution of pepper cultures and after subculture of the suspensions. The increase in PAL activity by elicitation and dilution was preceded by an increase in the amount of PAL mRNA detected by northern blot analysis which indicates de novo synthesis of the enzyme.